DOCTORAL THESIS

Effects of glucocorticoid overload on central regulatory systems involved in responses to stress – preclinical investigations into putative molecular targets in neuroimaging of stress-related mood disorders

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“Effects of glucocorticoid overload on central regulatory systems involved in responses to stress – preclinical investigations into putative molecular targets in neuroimaging of stress-related mood disorders”

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Abstract

Irregularities of the Hypothalamic Pituitary Adrenal (HPA) axis are implicated in stress-related mood disorders. The ensuing long-term elevations in circulating glucocorticoids are associated with neurobiological changes seen in depression. This thesis aims to identify some of the brain mechanisms by which exposure to chronic stress may lead to depression using a preclinical experimental approach.

The role of the serotonin system in the aetiology of mood disorders is well established, although this is not considered to be the only factor which causes these mood disorders. Interactions between the serotoninergic, peptidergic and endocannabinoid systems in response to glucocorticoids have been proposed. As all three neurotransmitter systems are involved in the regulation of the HPA axis, they are implicated in the dysfunction which is seen in depression. Furthermore, oxytocin, vasopressin and endocannabinoids are known to influence serotonergic neurotransmission and therefore it is pertinent to understand how glucocorticoids directly and indirectly impact serotonergic neurotransmission. In this thesis, the effect of chronic exposure to corticosterone on the serotonergic system is determined and also the relative contribution of the peptidergic and endocannabinoid systems to stress-induced mood disorders is considered. In addition, glucocorticoid-dependant receptor changes in these systems are related to neurotransmitter activity in brain regions involved in responses to stress. This has not previously been studied nor have the simultaneous effects of glucocorticoids on the serotonergic, peptidergic and endocannabinoid systems.

Here, preclinical approaches are applied to investigate the above mentioned receptor systems and their involvement in depression resulting from exposure to chronic stress. Administration of exogenous corticosterone (400 µg/mL) to rats for 21 days, via addition to drinking water, resulted in changes in expression of central 5-HT_{1A}, oxytocin, vasopressin 1a and CB1 receptors. This dose was selected as it has previously shown to induce depression-like behaviour in rats and also hippocampal atrophy similar to that seen in depressed patients (Magarinos et al 1998; Donner et al., 2012). In order to understand how these changes relate to the central concentrations of endogenous ligands, the concentration of serotonin, 5-HIAA, oxytocin and vasopressin was also measured in brain tissue.

Here, circulating corticosterone concentration was increased, as it is in chronic stress and in some depressed patients. Binding of the post synaptic 5-HT_{1A} receptor was upregulated in response to chronic stress in the form of elevated corticosterone concentration without a concomitant change in serotonin turnover suggesting that elevated corticosterone exposure modulates the 5-HT_{1A} receptor independently of serotonin turnover. Whereas presynaptic 5-HT_{1A} receptor binding was unaffected. In addition, chronic corticosterone exposure, as can be seen in depression, resulted in a decrease in binding to the oxytocin receptor in the hypothalamus associated with an increase in oxytocin concentration suggesting possible internalisation of the oxytocin receptor in this region. Conversely, there was an upregulation of the oxytocin receptor in the septal nuclei and raphé, with no associated change in oxytocin content in the same regions. Moreover, vasopressin 1a receptor binding was increased in septal nuclei and PODG subregion of the dorsal hippocampus, but decreased in the

hypothalamus. There was no change in vasopressin content in any brain region sampled, suggesting that these may be independent of peptide concentration. For the CB1 receptor, elevated corticosterone concentration, indicative of chronic stress, resulted in a decrease in receptor binding was found in the striata and raphé after chronic corticosterone treatment.

Taken together, in particular the effect on receptor binding in the raphé, the present data suggest that elevated corticosterone exposure may modulate serotonergic neurotransmission via the oxytocin and CB1 receptor. In addition, the hypothalamic peptidergic responses imply a potential role in glucocorticoid-induced dysregulation of the HPA axis. These changes may help further elucidate their respective roles in depression and stress related mood disorders.

In conclusion, the work presented in this thesis shows that the response to glucocorticoids is multifaceted and that there are changes observed in multiple neurotransmitter systems which regulate the HPA axis. Thus, the combined effect of the neurotransmitter systems studied here is of relevance to stress related mood disorders.

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Declaration

I declare that all the work in this thesis is original and my own work except where otherwise specified.

Rabia Ahmad

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Abbreviations

2-AG	2-Arachidonoylglycerol
5-HIAA	5-Hydroxyindoleacetic acid
5-HT	5-hydroxytryptamine
8-OHDPAT	8-hydroxy-2-(di-n-propylamino)tetralin
ACTH	Adrenocorticotrophic hormone
Ag	Silver
Ag+	Silver ion
AMP	Adenosine monophosphate
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
AVP	Arginine vasopressin
BBB	Blood brain barrier
BD	Bipolar disorder
BDNF	Brain derived neurotrophic factor
BP	Binding potential
BSA	Bovine serum albumin
CNS	Central nervous system
CORT	Corticosterone
COX 1,2	Cyclooxygenase 1 and 2
CRH	Corticotrophin releasing hormone
CSF	Cerebral spinal fluid
DAG	Diacylglycerol
DAGL	Diacylglycerol lipase
DALYs	Disability adjusted life years

DHEA	Dehydroepinadrosterone
DSM	Diagnostic and statistical manual of mental disorders
ECD	Electrochemical detector
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme linked immunosorbent assay
FAAH 1,2	Fatty acid amide hydrolase 1 and 2
fMRI	Functional magnetic resonance imaging
GABA	Gamma-aminobutyric acid
GR	Glucocorticoid receptor
HEK	Human Embryonic Kidney cells
HPA	Hypothalamic pituitary adrenal axis
HPLC	High performance liquid chromatography
i.c.v	Intracerebroventricular
i.p	Intraperitoneal
ICD	International classification of diseases
IFN-ϒ	Interferon gamma
IHC	Immunohistochemistry
IL-1,4,6,10	Interleukin 1, 4, 6 and 10
InsP3	Inositol phosphate 3
LTP	Long term potentiation
MAGL	Monoacylglycerol lipase
MAO	Monoamine oxidase
MBq	Mega becquerel
MDD	Major depression disorder
$MgCl_2$	Magnesium chloride
MRI	Magnetic resonance imaging
MTC	Mesiotemporal cortex
NAAA	N-acylethanolamine-hydrolyzing acid amidase
NICE	National institute for clinical excellence

NMDA	N- methyl-D-aspartate
NPY	Neuropeptide Y
OCD	Obsessive compulsive disorder
PACAP-27, 38	Pituitary adenylate cyclase activating polypeptide 27 and 38
PET	Positron emission tomography
PKC	Protein kinase C
POMC	Proopiomelanocortin
PSD-95	Postsynaptic density protein 95
PTSD	Post traumatic stress disorder
PVN	Periventricular nuclei
RIA	Radioimmunoassay
ROI	Region of interest
SBP	Serotonin binding protein
SERT	Serotonin transporter
SPE	Solid phase extraction
SPECT	Single photon emission computed tomography
SRTM	Simplified reference tissue model
SSRI	Selective serotonin reuptake inhibitor
TBq	Tera becquerel
THC	Tetrahydrocannabinol
TNF-α	Tumour necrosis factor -alpha
TRIS	Tris(hydroxymethyl)aminomethane
UV	Ultraviolet

Chapter 1

General Introduction

In 1965 Schildkraut and Ketty introduced the monoamine hypothesis for the aetiology of depression, which suggests that the disorder is a resultant effect of decreased concentrations of neurotransmitters, particularly monoamines (serotonin, noradrenalin and dopamine), in the brain (Raap et al., 1999). More specifically, the serotonergic system has been implicated in the aetiology of depression, as the major symptoms of the disorder, such as disturbances in mood, sleep and appetite, were found to be induced by a reduction in serotonin concentrations in the brain (Lucki, 1998). The theory focused on central serotonergic deficiency as a prominent feature in the aetiology of depression, which influenced the pharmaceutical industry towards developing drugs targeting the serotonergic system. This resulted in a wide-spread use of the narrowly selective pro-serotonergic antidepressants, namely serotonin selective

reuptake inhibitors (SSRIs) in the 1990's. However, since then it has been recognised that the aetiology of depression is more complex and efforts are now being placed on elucidating the contribution of other neuroregulatory systems that may be involved. Of particular concern is the high rate of patients who do not respond to currently available pro-serotonergic therapeutics (30-40%), which may be related to the heterogeneity of mood disorders (Kirsch et al., 2008; Matthews et al., 2005, Slattery and Neumann, 2010).

Considering that up to 50% of depressed patients exhibit underlying dysfunction of the hypothalamic pituitary adrenal axis (HPA), the contribution of elevated glucocorticoids towards depression and mood related disorders is of importance (Anacker et al., 2011). By understanding the involvement and interactions of other neurotransmitter systems, it is envisaged that advanced diagnosis and more effective drugs can be developed to improve the clinical management of depressed patients. To further this area of research, the influence of elevated circulating glucocorticoids on neurotransmitter systems involved in depression (its development and maintenance, and recovery from the disease) needs to be investigated and the potential to use these changes in stratifying and improving the therapeutic outcome of depressed patients needs to be evaluated.

1.1 Aims and Hypothesis

1.1.1 Aims

The aim of this thesis is to understand the influence of chronically elevated glucocorticoids on the serotonergic, peptidergic and endocannabinoid

neurotransmitter systems, which are known to be involved (or putatively involved) in the development of depression. More specifically, I aim to assess receptor responses to chronic glucocorticoid exposure, as a model of chronic stress, and couple these changes with an analysis of endogenous ligand concentration. Initially serotonergic responses to chronically elevated glucocorticoid level are determined as a reference system. Then the relative contribution of the oxytocinergic, vasopressinergic and CB1 receptor systems will be measured as there are known interactions between these neurotransmitter systems and serotonergic neurotransmission (Jørgensen et al., 2003; Haj-Dahmane and Shen, 2009; Yoshida et al., 2009). There is great interest in elucidating the role of oxytocin in the stress response as selective serotonin inhibitors (SSRIs) are known to induce the release of oxytocin (Uvnas Moberg et al., 1999). Oxytocin governs emotional behaviours that lead to positive social interactions (Kosfeld et al., 2005; Debiec 2007; Heinrichs et al., 2003) and is released in the hippocampus, amygdala, septum and hypothalamus i.e. regions that are rich in glucocorticoid receptors and involved in the regulation of mood (Engelmann et al., 1996; Huber et al., 2005; Liberzon and Young 1997). Furthermore the 5-HT_{1A} receptor antagonist WAY100635 inhibits 5-HT induced release of oxytocin and vasopressin (Jørgensen et al., 2003). Whereas, oxytocin has a tonic inhibitory role on the HPA axis, the vasopressinergic system is a potent activator of the HPA axis (Scott and Dinan, 1998). The effect of chronically elevated glucocorticoids on vasopressin 1a receptor binding in extralimbic brain regions has not previously been studied, although interactions between the vasopressinergic and serotonergic system are established. The V1a receptor is of relevance to aggression-related behaviour (McCall and Singer,

2012). Aggression is thought to be regulated via interactions between the serotonin and vasopressin systems, whereby serotonergic synapses have been observed on vasopressinergic neurones at the level of the hypothalamus, with serotonergic innervation to the hypothalamus originating from the raphé (Ferris and Delville, 1999). Thus, it is of interest to determine the vasopressinergic response to glucocorticoids in relation to those of the serotonergic and oxytocinergic systems. Furthermore, the study also aims to investigate CB1 receptor responses to corticosterone. The CB1 receptor also exerts a tonic inhibitory action on the HPA axis (Cota et al., 2007). Activation of the CB1 receptor in the raphé leads to inhibition of serotonin release in projection areas (Egashira et al., 2002) whereas pharmacological blockade of CB1 receptors enhances basal extracellular serotonin concentration in the prefrontal cortex (Haj-Dahmane, 2011). This is the first study to investigate the effect of corticosterone on CB1 receptor binding in extralimbic regions such as the raphé and attempts to relate any changes to the serotonergic and peptidergic system responses to glucocorticoids.

In addition, the present study aims to understand the glucocorticoid-induced changes in receptor binding in regards to endogenous neurotransmitter concentration. The changes observed in 5-HT_{1A}, oxytocin and vasopressin 1a receptor binding will be considered alongside changes in serotonin turnover, oxytocin and vasopressin content in brain regions involved in the stress response. This is the first time that this type of dual approach has been taken to gain a more complete understanding of the responses of these neurotransmitter systems to glucocorticoid induced dysfunction.

1.1.2 Hypothesis

It is hypothesised that chronic treatment with corticosterone, will lead to 5-HT_{1A} receptor density changes similar to those already published in the literature. More specifically, there will be a reduction in binding at the 5-HT_{1A} receptor in the limbic brain regions implicated in responses to stress. Also, it is hypothesised that these receptor changes will be linked to serotonin turnover effects in limbic brain regions implicated in responses to stress.

Also, it is hypothesised that chronic exposure to glucocorticoids will result in changes to the oxytocin and vasopressin 1a receptors in brain regions that are involved in the regulation of responses to stress. The direction of changes in receptor binding will reflect changes in the concentration of their endogenous neurotransmitter. In addition, it is hypothesised that plasma concentrations of oxytocin and vasopressin will reflect central concentrations of each neuropeptide.

With regards to the endocannabinoid system, it is hypothesised that chronic exposure to high concentrations of glucocorticoid for 21 days will result in changes to CB1 receptor binding in brain regions that are involved in the regulation of responses to stress. The direction of changes in receptor binding is expected to reflect changes in the concentration of endocannabinoids, according to the previously published literature. More specifically, it is hypothesised that chronic exposure to glucocorticoids will result in an decrease in CB1 receptor binding in amygdala and hippocampus amongst other brain regions associated with the regulation of responses to stress.

The aims and hypothesis stated above are tested using a preclinical approach, whereby rats were administered corticosterone (400 µg/mL) for 21 days and

changes in receptor binding were assessed using autoradiography. Furthermore, concentrations of serotonin, oxytocin and vasopressin were measured in discrete brain regions using HPLC or radioimmunoassay. Exact details of methodology are given in chapter 4 and further discussion of the aims and hypothesis are given in the respective experimental chapters. As hypothesised above, chronic exposure to glucocorticoids will result in changes in the serotonergic, peptidergic and endocannabinoid systems which all regulate the stress response. There are interactions between these neurotransmitter systems as well as their involvement in the stress response. It is hypothesised that glucocorticoid exposure influences the serotonergic system, but this is also affected by the peptidergic and endocannabinoid systems which could contribute to the dysfunction observed in stress-related mood disorders.

1.2 Outline of thesis

This thesis is organised in the following manner:

In chapter 2, an introduction to depression, anxiety and hence stress related mood disorders is given. I discuss the factors that cause these disorders and the current understanding of the neurobiology of depression with a focus on the involvement of HPA axis dysfunction. Then in chapter 3, I discuss the involvement of the central serotonergic, peptidergic and endocannabinoid systems in depression and regulation of the stress response.

Chapter 4 focuses on methodology. I discuss the theory of techniques selected for use in this thesis with justification for their selection. I then provide detailed methods for the experiments conducted as part of this investigation. I

also report findings from a feasibility study which was designed to evaluate the most effective method for dosing rats with glucocorticoids for subsequent experimental chapters, i.e. administering corticosterone (CORT) by daily subcutaneous injections or addition of CORT to drinking water for 21 days.

Chapters 5, 6 and 7 describe the experimental studies designed to assess whether elevated corticosterone concentrations induce changes in central receptors or their endogenous ligands. Changes are measured in discrete brain regions known to be involved in the regulation of mood. Chapter 5 focuses on the serotonergic system. I report on changes in binding to the 5-HT_{1A} receptor and attempt to relate them to changes in endogenous serotonin concentration. In chapter 6, I describe changes in binding to the oxytocin and vasopressin 1a receptors in response to elevated glucocorticoid levels and also attempt to relate them to changes in endogenous peptide concentrations. Chapter 7 describes changes at the CB1 receptor after chronic exposure to corticosterone.

Finally, chapter 8 summarises the findings from the work presented herein with a view to understanding the impact of glucocorticoids on serotonergic and non-serotonergic receptor responses and coupling these with an analysis of endogenous ligand concentration in both limbic and extralimbic regions. The interactions of these neurotransmitter systems are also discussed. I also outline future work that is enabled from this study and the potential of these receptor systems as targets for molecular imaging of glucocorticoid associated changes in mood disorders.

Chapter 2

Depression and anxiety as stress related disorders

Mental health disorders such as depression, unipolar or bipolar disorder, depressive illness and dysthymia are common and widespread throughout the world. It is estimated that 1 in 6 people will experience some form of depression at any given time in the UK, which is often accompanied by an anxiety –related disorder (The Office for National Statistics, 2000). Depression is the most costly mental health disorder in Europe, with an annual spend of € 118 billion in 2004 as determined from a population of 466 million, across 28 countries (Sobocki et al., 2006). Furthermore, treatment resistant patients show an even higher per patient medical cost (Olchanski et al., 2013). In fact, The World Health Organisation (WHO) have reported that the incidence of depression is on the increase and that this disorder could come second place in the ranking of Disability Adjusted Life Years (DALY's, calculated as the sum of years of

potential life lost due to premature mortality and the years of productive life lost due to disability) for all ages and both sexes worldwide by the year 2020 This warrants further research into the causes, treatment and prevention of depression in order to curb its global growth and its associated burden on the economy (Murray and Lopez 1997; WHO, 2012).

2.1 Different types of depression

Depression is a heterogeneous disorder with patients experiencing a varying degree of depression from a single episode, to one which may become progressively worse, or to those that follow a remitting-relapse pattern throughout their lives. Each form of depression is distinct from each other, although there may be some overlapping symptoms. Major depressive disorder (MDD) is characterised by a series of symptoms which significantly affect an individual's ability to function normally. Daily activities such as sleeping, eating and working are severely disrupted. Dysthymic disorder is not as severe as MDD, but the symptoms will last for at least two years, although episodes of MDD may co-occur during this time. There are also a range of disorders which can be grouped and classified as minor depression; these are psychotic depression, postpartum depression and seasonal affective disorder. In addition, there is a less common type of depression called bipolar depression (BD) which is characterised by severe mood swings, from extreme high and lows (National Institute of Mental Health, 2011). Depression may also arise as a result of suffering a life-changing illness such as cancer (Avis et al., 2013), diabetes (Baumeister et al., 2012) and stroke (Bartoli et al., 2013) amongst many chronic illnesses which may lead to depression.

Depression is often comorbid with an anxiety related disorder and in fact, prolonged periods of anxiety can lead to depression. Anxiety disorders are also heterogeneous ranging from generalised anxiety disorder, phobias, panic disorder, post-traumatic stress disorder (PTSD), obsessive compulsive disorder (OCD) and separation disorder (Kessler et al., 1995). One of the major risk factors for developing an anxiety disorder is exposure to stress (Yehuda, 2002). The neurobiology of depression and stress-related depression can be found in section 2.1.3.

2.1.1 Diagnosis of depression

As a diagnostic test for depression does not exist, clinicians depend on the use of patient self-reports and clinical observations, which are scored against a recognised set of behavioural and clinical criteria stated in the Diagnostic and Statistical Manual of Mental Disorders (DSM, American Psychiatric Association), the International Classification of Disease (ICD, World Health Organization) and guidance from the National Centre for Health and Clinical Excellence (NICE 2009). However, diagnosis in this way can be subjective, nonspecific and many clinicians struggle to confirm a diagnosis of depression which has led to the over prescription of antidepressants into the general population (Pajer et al., 2012). In addition, a further clinical unmet need is for a test which distinguishes between the different types of depression. This would enable the stratification of patients according to the type of depression, allowing for more effective selection of treatment regime and hopefully a better therapeutic outcome.

Interest is growing in identifying biomarkers which may improve the accuracy of diagnosis providing objective data to confirm the verbal information provided by the patient, or specificity of the diagnosis to a particular type of depression (Pajer et al., 2012). A biomarker is defined as a measurable feature that is associated with a particular disorder (Pajer et al., 2012). They can be used for diagnosis or stratification of patients within a heterogeneous disease and determining the most suitable treatment approach (Schmidt et al., 2011). Whilst it is accepted that no single biomarker exists for depression, investigators continue to study changes in depression and look for a biosignature or panel of biomarkers which may aid diagnosis or stratification of patients for therapy. These can be classified as either blood based cerebrospinal fluid (CSF) or tissue based biological changes that are associated with a disease and can be measured using a range of techniques (detailed in chapter 4).

A range of variables have been investigated as potential biomarkers from inflammatory mediators, cytokines and growth factors through to hormones and receptors (Schmidt and Duman, 2007; Krishnan and Nestler 2008; Miller et al, 2009; Castren and Rantamaki, 2010). For example, brain-derived neurotrophic factor (BDNF) has been reported to be reduced in the blood of depressed patients (Sen et al., 2008); and concentrations of monocyte chemoattractant protein-1 are increased in cerebral spinal fluid (CSF) of depressed patients. Moreover, the serotonin metabolite, 5-hydroxyindole acetic acid (5-HIAA) is a strong predictor of depression (Raison et al., 2009), and glucocorticoid levels in plasma have been shown to reflect central changes in glucocorticoid receptor expression in some, but not all depressed patients (Schmidt et al., 2011).

Non-invasive molecular imaging techniques such as positron emission tomography (PET) and single photon emission computed tomography (SPECT) can be used to detect abnormalities in the brains of depressed patients, such as changes in receptor number or affinity. These receptor changes can be considered as biomarkers of the disease they are associated with and are discussed further in section 2.1.3. At present, PET/SPECT can be used as a diagnostic tool for neurological disorders, as a research tool to understand the neurobiology of the disorder and also as a pharmaceutical tool to measure drug receptor occupancy during development of novel therapeutics. GE Healthcare is particularly interested in the development of radiolabelled probes to potentially diagnose, stratify and monitor treatment responses in depressed patients. However, in order to do so, neurobiological changes which are commensurate with PET/SPECT imaging need to be identified before radiolabelled probes can be developed to target these. Thus, a greater understanding of the aetiology and neurobiological changes associated with depression is needed.

2.1.2 Factors causing depression

Although the precise aetiology of depression is not fully understood, it is widely accepted that environmental, genetic and neurobiological factors will affect the onset of the disease. Genetic studies conducted in twins have identified a heritable link in depression (Ørstavik et al., 2007; Kendler et al., 2009; Sullivan et al., 2000). It has been suggested that at least 37% of major depression is heritable, whereas the prevalence of BD in the general population is 1%, but can be up to 20% in first degree relatives of the BD subpopulation (Merikangas et al., 2002) and 60% in monozygotic twins (Kendler et al., 1993). Furthermore,

not all mood disorders share the same heritability. Early-onset, reoccurring and severe depression have higher heritability than other forms (Kendler et al., 2009). Most of the research on a familial link to depression has focused on identifying genetic polymorphisms which occur within families, however, contradictory results have been reported (Arias et al., 2012; Illli et al., 2013). Although a single gene mutation responsible for the development of depression has not yet been identified, areas of chromosomes with a link to depression have been found. Mutations in these areas affect monoamine neurotransmission and cause polymorphisms of other neurochemicals such as BDNF as well as over activation of the hypothalamic pituitary adrenal (HPA) axis (Levinson 2006).

Another common risk factor for development of depression is exposure to stress for example childhood abuse, early life neglect, a stressful life event (Kendler et al., 2008; Heim and Nemeroff, 2010). The majority of the general population will have experienced extreme stress at some point in their lives and considering that anxiety-related disorders and mood disorders have a combined lifetime prevalence of 49.6%, with an early onset (median age of 11 years and 30 years respectively), a vast proportion of the population are at risk of developing one of these (Kessler et al., 2005). Many studies have correlated exposure to stress with development of depression (Turner et al., 1995; Turner and Lloyd, 1999). A greater frequency of stressful life events leads to an increase in depressive symptoms (Gibbs and Rude, 2004) and chronic stress is more strongly related to depression than acute stress (McGonagle and Kessler, 1990). The type of stressors that may lead to the onset of a mood disorder is variable; from the birth of a child, the death of a loved one, physical abuse, financial

worries and work-related stress. The mechanism of how stress leads to depression is discussed later in section 2.2

2.1.3 Neurobiology of depression

Depressive disorders are known to be associated with structural and neurochemical changes in the brain, which may represent neuroplastic changes. Neural plasticity is the mechanism by which the brain circuitry is able to adapt and reorganise itself in response to internal and external stimuli such as life experiences or brain injury (Duman, 2009). Neuroplastic changes may manifest as changes in dendritic function, synaptic remodelling, long term potentiation, axonal sprouting, neurite extension, synaptogenesis and neurogenesis (Manji et al., 2003). In this thesis, plasticity is determined as the ligand dependant up- or down regulation in receptor expression as these are changes which may be imaged by PET/SPECT.

The regions of the brain which regulate emotions, mood and associated behaviours is collectively called the limbic system. This is a group of structures that links autonomic responses to behavioural responses. The main regions of the brain which comprise the limbic system are the cingulate, amygdala and hippocampus. These limbic regions are connected to the forebrain regions and also the hypothalamus. The hippocampus primarily functions in learning and memory processes receiving inputs principally from the entorhinal cortex, and projecting to the septal area (Nolte, The Human Brain, 5th edn). The amygdala functions as the emotional processing centre of the brain and receives afferents carrying a huge amount of sensory data from several locations and projects to the cerebral cortex and hypothalamus (Nolte, The Human Brain, 5th edn). In

addition to the limbic system, aberrant function of the prefrontal cortex is also evident in depression. This region functions to control cognition and working memory (Duman, 2009).

2.1.3.1 Neuroimaging in depression

Structural abnormalities in the limbic region of depressed patients can be visualised using neuroimaging and *post mortem* analysis. In depression, hippocampal volume reductions of between 10-20% have been reported in depressed patients measured using MRI (Bremner et al., 2000; Duman, 2009). However, amygdala volumes have been observed to either increase (Frodl et al., 2003) or decrease in depression (Sheline et al., 1998; Kronenberg et al., 2009). In addition, *in vivo* MRI imaging studies have revealed a reduced volume of the prefrontal cortex in depressed patients (Reviewed in Duman. 2009). These structural abnormalities may be specific to the subtype of depression, as reductions in the prefrontal cortex and amygdala showed specificity to BD when compared with MDD (Brambilla et al., 2002; McGrath et al., 2004).

Post mortem studies also show structural abnormalities associated with depression. A reduction in hippocampal volume is associated with the length of time the subject suffered from depression (Cobb et al., 2012). Surprisingly, although hippocampal volume is reduced, there is an increase in the density of glia, pyramidal neurons and granule cell neurons (Stockmeier et al., 2004). In addition, *post mortem* studies have revealed changes in the forebrain of depressed patients, with a concomitant decrease in cortical thickness, glial density, neuronal size and density (Rajkowska et al., 1999).

Further to these structural changes, functional neuroimaging (PET, SPECT, fMRI) of depressed patients has allowed for the indirect measurement of abnormalities in regional brain activity (McGrath et al., 2004). This can be determined by measuring changes in blood flow, glucose metabolism or receptor binding. In depression, there is a reduction in cerebral blood flow to the prefrontal cortex, frontal cortex and limbic brain regions (Bonne et al., 1996; Ito et al., 1996; Drevets et al., 1997). There is also a general and global reduction in glucose metabolism in depressed patients; however, some studies have revealed an increase in glucose metabolism in the prefrontal cortex and limbic system (Baxter et al., 1989; Ketter et al 2001). In addition, fMRI studies have also shown increased activation of the limbic regions in depression (reviewed in McGarth et al., 2004).

There are only a small number of radioligands currently available for imaging depression. Most of these have focused on the serotonergic system, where changes in binding to the 5-HT_{1A}, 5-HT_{2A}, 5-HT_{2C} receptors and also the serotonin transporter have been reported in depression. However results from these studies are inconsistent and contradictory (more details are described in chapter 3). Imaging studies on other neurotransmitter systems are limited; however PET imaging of the dopaminergic system has revealed a reduction in dopamine D_1 receptor binding in the striata of depressed patients. Whereas, $D_{2/3}$ receptor binding is just as inconsistent as the serotonergic changes reported. Furthermore, a reduction in binding to the histamine 1 receptor and the muscarinic M2 receptor was seen in depression, but these receptor systems are less well studied in depression.

2.1.3.2 Neurochemistry of glucocorticoid related depression

The neurochemistry of depression is complex and involves many different neurotransmitter systems. These are discussed further in chapter 3. However, up to 50% of depressed patients also show an elevation of circulating cortisol levels throughout the circadian rhythm. This is often regarded as a predisposing risk factor for developing a psychiatric disorder (Keller et al., 2006; Anacker et al., 2011; Pillai et al., 2012). Cortisol is a glucocorticoid and also the primary mediator of the stress response. It is produced in response to the activation of the HPA axis. More details of the stress response and the HPA axis are given in section 2.2. However, amongst many functions, glucocorticoids are involved in neurobiological processes such as neurogenesis, neuroinflammation, neurodegeneration, memory and learning (Garcia-Bueno et al., 2008). Importantly, glucocorticoids enhance mood, emotions and mental performance, all of which are adversely affected in depression (Lupien et al., 2007; de Kloet et al., 2008).

Preclinical studies have shown that corticosterone (the rodent equivalent to cortisol) is required for the formation of memories, maintenance of working memory and extinction of those memories no longer required (de Kloet et al., 2008). However, exposure to chronic stress results in a deficit in learning and memory tasks (McEwen 1999; McLaughlin et al., 2007) and also enhances fear conditioning by strengthening adverse memories which arise from stress-induced sensitisation of the amygdala (Sandi et al., 2001). In addition, chronic exposure to corticosterone or repeated stress induces atrophy of apical dendrites in the CA3 subfield of the hippocampus characterised by decreases in branch points, branch length and synaptic suppression which is associated with loss of spatial

learning and memory (Bisagno et al., 1999; Magarinos and McEwen 1995; Magarinos et al., 1998; McEwen, 2005; McLaughlin et al., 2007; Sousa et al., 2000). These appear to be reversible when glucocorticoid levels are returned to normal (Duman. 2009). Importantly, these glucocorticoid induced changes in brain are similar to the changes that have been observed in *post mortem* brains of depressed patients (Manji et al., 2003).

Moreover, specificity of HPA axis dysfunction (and hence hypersecretion of glucocorticoids) to depression comes from observations that antidepressant treatments normalise many aspects of the HPA axis dysfunction, thus providing further evidence of its involvement in the development of depression. For these reasons, it is thought that there is a significant contribution of HPA axis dysfunction in depression, and therefore the regulation of this is extensively studied.

2.2 Stress

Physiological stress was defined by Selye in 1936 and is the term given to a phenomenon which alters the normal functioning of the body (Selye 1936). Stress can be defined as a real or perceived threat to an individual's wellbeing (McEwen 2000). Organisms have developed different physiological responses to stress that are essential for the maintenance of life. The theory of *Hawks* and *Doves* personality types described by Korte et al., 2005 states that the 'Hawks' show high aggression and are more likely to be involved in flight-or-fight responses, whereas 'Doves' show low aggression and so when faced with a stress will exhibit a freeze-or-hide response. The physiological response to stress can be protective or adaptive, for example, Sapolsky (2000) famously described

a scenario in which a zebra running away from a predatory lion will need to divert energy away from physiological functions which are not essential at that particular moment in time i.e. suppression of appetite, immune response, gastrointestinal activity, reproductive and growth systems (protective measures) to enable energy to be diverted for adaptive responses such as increased gluconeogenesis, lipolysis, proteolysis, blood pressure, breathing rate and heart rate. At the end of the stress response the normal physiological and biochemical state is restored. Although this system is highly regulated, it can lead to disease if it is chronically activated especially in response to continuous psychological, social and cognitive stimuli.

2.2.1 Homeostasis, allostasis and allostatic load

There are certain parameters which are crucial for survival and these are required to be kept constant within a very narrow range e.g. pH, temperature, osmolarity and oxygen tension. The mechanism by which the body maintains this is called homeostasis (McEwen 2000). However, there are some physiological parameters, which are able to adapt to a changing environment. For example, heart rate, blood pressure, synthesis and release of some cytokines, neurotransmitters and hormones. The flexibility of these critical systems helps maintain essential-to-life parameters relatively constant, a process which is called allostasis (Karatsoreos and McEwen, 2011). The mediators of allostasis are listed in table 2.1. The physiological concentration of these mediators is tightly regulated, and when dysregulated, results in structural and chemical changes that may lead to pathological changes. This damage is termed allostatic load i.e. the cost (negative effects) of adaptation (McEwen 2002a and b; 2003).

An allostatic load may occur after exposure to repeated stress, failure to habituate to repeated stress, failure to turn off the stress response after a threat has passed or through the inability to produce an adequate response.

2.2.2 Hypothalamic-Pituitary-Adrenal Axis

The hypothalamic pituitary adrenal (HPA) axis is a neurological mechanism which serves to connect the nervous and endocrine systems, extending across the hypothalamus, pituitary gland and adrenal glands. Its primary function is the secretion of glucocorticoids, cortisol in humans and corticosterone in rodents, and also the secretion of adrenaline and noradrenaline (Lupien et al., 2007).

Table 2.1 – Table identifying mediators of allostasis. Adapted from McEwen 2002(b).

Mediators of allostasis
Systemic mediators Glucocorticoids DHEA (5-Dehydroepiandrosterone) Catecholamines (adrenaline, noradrenaline) Cytokines (e.g. IL-6, IL-1, TNF-α) Many systemic hormones (e.g. thyroid hormone, insulin, insulin-like growth factors, leptin) Many pituitary hormones (e.g. prolactin, ACTH, growth hormone)
Tissue mediator Cortico-Releasing Factor Excitatory amino acids Monoamines (e.g. serotonin, noradrenaline, adrenaline, histamine) Neuropeptides (e.g. oxytocin, vasopressin, neuropeptide Y, cholecystokinin, enkephalin, dynorphin, substance P Other neurotransmitters (e.g. GABA, glycine endocannabinoids)
Others Many cytokines (e.g. TNF-α, IL-6, IL-4, IL-10, IFN-γ) Some pituitary hormones (e.g. prolactin, POMC)

Glucocorticoids function to maintain homeostasis. Upon activation of the HPA axis, the parvocellular neurosecretory cells of the paraventricular nuclei in the hypothalamus release corticotroph releasing hormone (CRH) and also the neuropeptide arginine vasopressin (AVP) into the capillaries of the primary plexus. CRH and AVP travel in the hypophyseal portal veins to the anterior pituitary gland (Raison and Miller 2003). Here, they induce the release of adrenocorticotroph hormone (ACTH) into the secondary plexus and hypophyseal veins and eventually into the general circulation. ACTH acts on the adrenal glands to stimulate the production of glucocorticoids. Once released, glucocorticoids exert a range of effects associated with maintenance of homeostasis and affect a wide range of cellular and molecular networks through transcriptional influence over a large percentage of the human genome (Chrousos and Kino 2007). Glucocorticoids are highly lipophilic and readily cross cell membranes. The hormone binds to intracellular receptors (these are discussed later), upon binding the receptor complex translocates to the nucleus where it interacts with transcription factors or binds to DNA directly resulting in an up- or down-regulation of the expression of certain genes (Pariante and Miller 2001). This is depicted in figure 2.1.

Glucocorticoids, as mediators of allostasis, regulate a number of important physiological processes, such as mood, sleep, metabolism, normal cardiovascular tone and activity of the immune system (Chrousos and Kino 2007). Because of this, they are also responsible for allostasis and allostatic load resulting in disease. Hence, chronically elevated concentrations of glucocorticoids are involved in many pathologies; e.g. cardiovascular disease, diabetes, hypertension, osteoporosis, autoimmune inflammatory disease, allergic

disorders and depression (Swaab, 2005; Lupien et al., 2007). There are 'checks and balances' to maintain the status quo and prevent an allostatic load e.g. glucocorticoids promote gluconeogenesis and then initiate an increase in locomotor activity and appetite to replenish the diminished energy stores. However, if appetite is increased without an accompanying increase in locomotor activity, a state of allostatic load is attained. The individual is then at risk of obesity, cardiovascular disease, insulin resistance or loss of bone mineral density and muscle protein (McEwen 2002b). Thus, it is important to regulate the concentration of glucocorticoids. Thus, the HPA axis is tightly controlled by a sensitive negative feedback mechanism which is regulated by circulating glucocorticoids. This is discussed in more detail in section 2.2.1.

As glucocorticoids are lipophilic, they readily cross the blood brain barrier to exert their effects through interactions with two intracellular receptors, the high affinity mineralocorticoid receptors (MRs) (Kd = 0.5nM) and the lower affinity glucocorticoid receptors (GRs) (Kd = 5nM) (Rozeboom et al 2007). These receptors are densely located in important regions for regulating processes associated with learning, memory and emotions; namely, the hippocampus, amygdala and frontal cortex (Lupien et al 2007). When the release of glucocorticoids is blocked, these processes are impaired (Maheu et al., 2004; Lupien et al., 2007). Glucocorticoid secretion follows a circadian rhythm, which peaks during the morning, slowly declining to its lowest level during the nocturnal period (circadian trough; evening in humans but morning in rats) (Lupien et al., 2007).

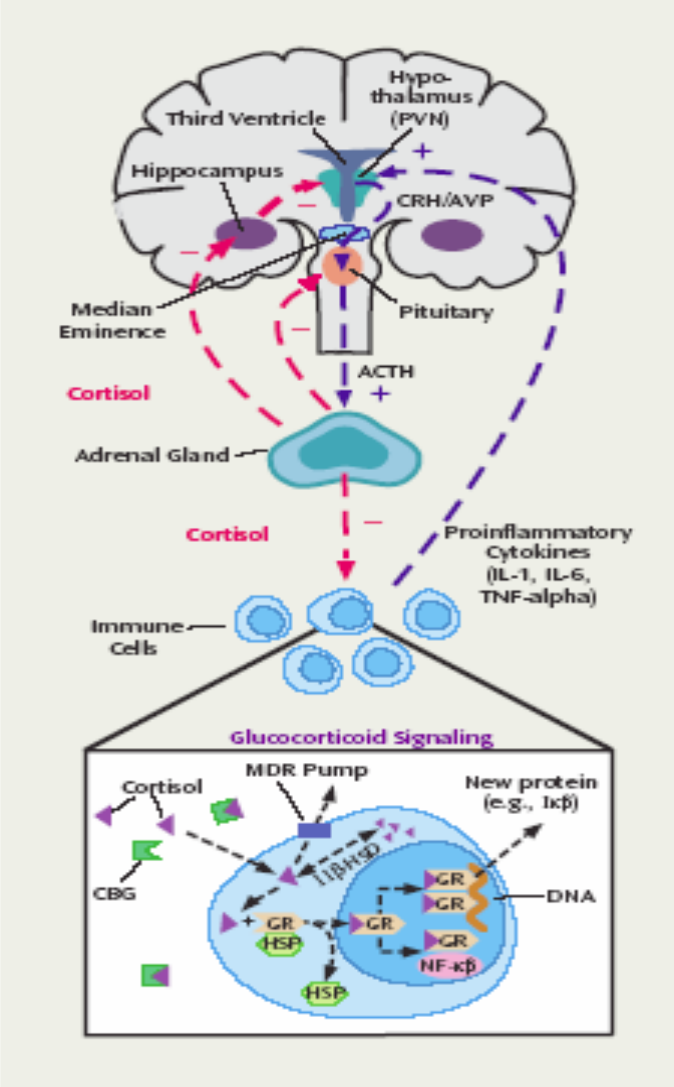


Figure 2.1. Pathways of glucocorticoid signalling. Taken from Raison CL and Miller AH (2003). Reprinted with permission from the American Journal of Psychiatry, (Copyright ©2003). American Psychiatric Association.

A high density of the MRs is also found in the hypothalamus, a region of the brain that is primarily involved in homeostatic control and a high density is also found in the hippocampus. In contrast, the glucocorticoid receptors are widespread throughout the brain, but highly expressed in hypothalamic CRH neurons and pituitary corticotrophs as well as the amygdala and prefrontal cortex (Herman et al., 2005). They co-localise with MRs in the hippocampus but have a much lower affinity (6-10 times lower) for corticosterone (Han et al., 2005;

Lupien et al., 2007). Thus, glucocorticoids have a tonic influence over the HPA axis via MRs such as during the circadian trough. In this phase, approximately 90% of MRs are occupied and only 10% of GRs (Lupien et al., 2007). Whereas GRs only come into play at much higher concentrations of glucocorticoids, e.g. those observed after a prolonged period of stress (Spencer et al., 1998), or during the circadian peak, where the MRs are saturated and approximately 67-74% of GRs are also occupied (Lupien et al., 2007). Therefore, it is the GRs which regulate the feedback inhibition of the HPA axis (as discussed further in section 2.2.3).

The continuous control of the HPA axis employs the involvement of numerous neurotransmitter systems/receptors including the above mentioned glucocorticoid and mineralocorticoid receptors, the serotonergic system, the neuropeptides; oxytocin, vasopressin and the endocannabinoid system. In particular, interactions between the glucocorticoids and serotonergic systems have been determined to be pivotal in the regulation of the HPA axis. Serotonin activates the HPA axis by acting at the hypothalamus to impact CRH release. Enhanced serotonergic neurotransmission has led to increased glucocorticoid release into the bloodstream (Heisler et al., 2007). Furthermore, electron microscopy has shown interactions of 5-HT neurone axons and CRH-containing neurones in the paraventricular nuclei (PVN) indicating a direct influence of serotonin on activation of the HPA axis (Liposits et al., 1987). In addition, there are two important neuropeptides which regulate the HPA axis; vasopressin and oxytocin. Vasopressin functions synergistically with CRH to activate the HPA axis. Oxytocin on the other hand has an inhibitory tone on the HPA axis, possibly by activating HPA re-activity (Neumann et al., 2000). More recently,

there has been a lot of research around the involvement of endocannabinoids in the HPA axis. Each of these receptor systems and their respective roles in the stress response and depression are described in more detail in chapters 2 and 6-8.

2.2.3 Regulation of the HPA axis – Negative feedback mechanism

Under normal conditions, the concentration of glucocorticoids increases in response to a stressful stimulus. However, in order to 'switch off' this stress response, the HPA axis negative feedback mechanism must be activated, see figure 2.2. This is triggered by the increase in glucocorticoid concentration and prevents the further release of CRH, ACTH and hence glucocorticoid. In this way the organism is able to protect itself from an allostatic load. The negative feedback of the HPA axis is primarily mediated through the glucocorticoid receptor (Falkenberg and Rajeevan 2010).

The glucocorticoid induced negative feedback mechanism is a coordinated response involving different brain regions. The bed of nucleus stria terminalis (BNST), amygdala and hippocampus all exert an inhibitory tone on the HPA axis. The BNST is an important region that integrates and processes both excitatory and inhibitory signals from limbic regions i.e. there are GABAergic projections from the amygdala to the anterior BNST and from the posterior BNST to the hypothalamus. Specific lesions in either the anterior or the posterior BNST result in increased or decreased corticosterone secretion, respectively (Dunn and Whitener 1986; Dunn, 1987). The amygdale receives excitatory inputs from cortical and thalamic regions and is connected to the hypothalamus, but also to other regions which also project to the hypothalamus.

The hippocampus is the primary mediator of the negative feedback mechanism and hence has a high density of glucocorticoid and mineralocorticoid receptors. Inhibition of the HPA axis from the hippocampus occurs via glutamatergic efferents from the ventral subiculum to the dorsal medial hypothalamus where they make contact with GABAergic neurones that inhibit the activation of the negative feedback mechanism in the PVN (Bowers et al., 1998).

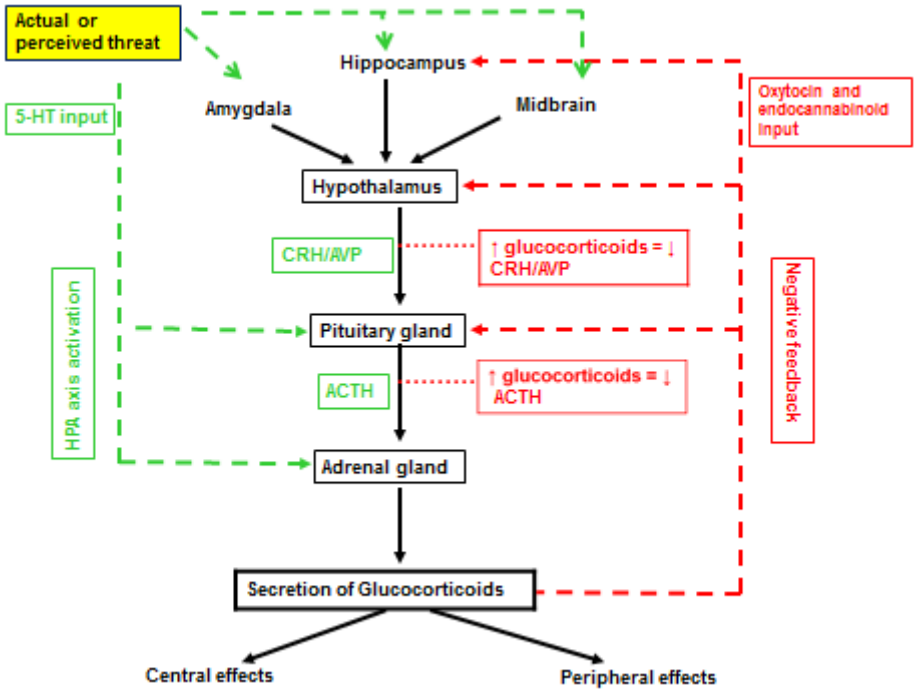


Figure 2.2. Schematic diagram outlining the HPA axis and negative feedback mechanism. The black arrows represents the normal functioning of the HPA axis and a common pathway where, CRH=corticotrophin releasing hormone, AVP=arginine vasopressin, ACTH=adrenocorticotroph hormone. The green pathways represents inputs that activate the HPA axis where, 5-HT=serotonin. The red lines represent the negative feedback mechanism which inhibits the HPA axis.

2.2.4 Diseases associated with HPA axis dysfunction

Irregularities of the negative feedback mechanism can lead to either hyperactivity or hypoactivity of the HPA axis. The glucocorticoid cascade

hypothesis describes a hyperactive HPA axis whereby, prolonged exposure to stress is associated with disruption of the normal feedback mechanism resulting in the overproduction of glucocorticoids or it could arise from a genetic predisposition to HPA axis hyperactivity (Raison and Miller, 2003). This leads to a feed-forward mechanism whereby the concentration of glucocorticoids continues to increase resulting in an allostatic load. Conversely, HPA axis hypoactivity results from insufficient glucocorticoid production (Raison and Miller, 2003). Table 2.2 below lists examples of various clinical states that have been associated with disrupted HPA function. Interestingly, glucocorticoid therapy to overcome deficiencies induces steroid psychosis, suggesting that an exogenously induced elevation in glucocorticoid concentration results in neurobiological changes that develop into psychiatric illness (Lupien et al., 2007).

Table 2.2 – Medical states associated with HPA axis dysfunction. Adapted from Juruena et al., 2004.

Increased HPA axis activity	**Decreased HPA axis activity**	**Disrupted HPA axis activity**
Severe chronic disease	Atypical depression	Cushing syndrome
Melancholic depression	Seasonal depression	Glucocorticoid deficiency
Anorexia nervosa	Chronic fatigue syndrome	Glucocorticoid resistance
Obsessive-compulsive	Fibromyalgia	
Panic disorder	Adrenal suppression	
Chronic excessive exercise	Post stress	
Malnutrition	Hyperthyroidism	
Diabetes mellitus	Rheumatoid arthritis	
Central obesity	Nicotine withdrawal	
Pregnancy	Postpartum	
	Menopause	

One of the most prevalent disorders of a hyperactive HPA axis is depression. There is extensive evidence demonstrating that depressed patients exhibit increased levels of circulating glucocorticoids, increased glucocorticoid excretion, decreased corticosteroid receptor function, decreased response to ACTH by the pituitary gland, an enhanced adrenal response to ACTH and increased levels of CRH in cerebrospinal fluid (CSF), plasma and urine (Holsboer, 1999; Chrousos and Kino, 2007; Swaab et al., 2005; Juruenas et al., 2004).

In the next chapter, I discuss the role of the serotonergic, neuropeptidergic (oxytocin and vasopressin) and endocannabinoid neurotransmitter systems in stress related depression. Then in the remainder of the thesis, I outline studies designed to investigate the effect of chronic exposure to glucocorticoids on these neurotransmitter systems with a view to identify the value of imaging these in depression.

Chapter 3

Neurotransmitter systems involved in stress related depression

It is well established that depression is a multifactorial disease which involves perturbations in many neurotransmitter systems. In particular, when considered as a stress related disorder, irregularities in the systems that regulate the HPA axis are implicated. This thesis focuses on the neurotransmitter and receptor specific responses of the serotonergic, neuropeptidergic and endocannabinoid systems to elevated glucocorticoid levels with a view to determine whether any such changes/responses could be applicable to imaging stress related depression using PET. In this chapter, I first describe the current strategies towards PET imaging of depression, and then outline the role of each of the neurotransmitter systems mentioned above in the regulation of the stress response and abnormalities that have been reported in depression.

3.1 Introduction

For many decades, irregularities in the monoamine and then later on the serotonergic system were considered to be responsible for the development of depression. Traditionally it was thought that depression is associated with reduced serotonergic tone in the brain, whereas anxiety is associated with increased serotonergic activity (reviewed in Jacobsen et al., 2012). However, this assumption now stands inaccurate as a serotonin deficit is not seen in all cases of depression and the fact that between 30-40% of patients do not respond to pro-serotonergic antidepressant therapies (Matthews et al., 2005; Jacobsen et al., 2012). Instead, depression is now considered to be multifactorial, and is thought to arise due to dysregulation of many different pathways (Jacobsen et al., 2012). This is especially true if the heterogeneity of depression is considered. As outlined in chapter 2, there are many factors which can lead to the development of depression, however an underlying common pathway which is seen in up to 50% of depressed patients is HPA axis hyperactivity (Anacker et al., 2011). The resultant elevation in cortisol concentration throughout the diurnal rhythm contributes to neurobiological changes associated with depression. Thus the irregularities in the mechanisms which govern the HPA axis are implicated in the aetiology of depression. In particular the serotonergic, peptidergic and endocannabinoid systems have been investigated for their role in the onset and development of the disorder. A detailed discussion on each of these can be found in sections 3.2, 3.3 and 3.4 respectively.

Clinically, neurobiological abnormalities in depression can be studied *in vivo* using PET. In this respect, the serotonergic system has received the most attention to date. However, such studies have revealed contradictory results,

confounding the interpretation of these and failing to find a relationship between serotonergic dysfunction and severity of depression. There are numerous radioligands available for imaging SERT, 5-HT_{1A} and 5-HT_{2A} receptors. Some PET studies have reported a reduction in 5-HT_{1A} receptor binding potential (BP) in mesiotemporal cortex (MTC) by 27%, and raphé, by 42%, in unmedicated-depressed patients (Drevets et al., 1999). Similar results have been reported for the insula, cingulate cortex, temporal and orbital cortex (Sargent et al., 2005). In addition, a 17 % decrease in 5-HT_{1A} receptor binding was measured in drug-free, recovered depressed patients (Bhagwagar et al., 2004). However, in contrast, others have shown an increase in 5-HT_{1A} receptor binding in drug naïve or recovered depressed patients (Parsey et al., 2006; Sullivan et al, 2009), and there is one report of a PET study that showed no difference in the binding to 5-HT_{1A} receptors of depressed subjects (Mickey et al., 2008). The contradictory results obtained from these studies can be attributed to differences in the subpopulation of depressed patients selected, effects of medication and also differences in the analysis of the PET scan. For example, the use of the simplified reference tissue model (SRTM), with the cerebellum as the reference region, may result in an underestimation of BP due to the presence of a significant number of 5-HT_{1A} receptors in the cerebellum (Parsey et al., 2006).

Such discrepancies have also been noted in studies that attempt to image dysfunction in SERT and the 5-HT2 receptors. Both increases (Reivich et al., 2004; Cannon et al., 2006a) and decreases (Parsey et al., 2006; Oquendo et al., 2007) in the expression of the serotonin transporter in depressed patients have been reported. The same was observed when imaging the 5-HT2 receptors (Meyer et al., 1999; Sheline et al., 2004; Bhagwagar et al., 2006). Again, the

differences may be attributed to the different subset of depressed patients used in these studies.

With regards to molecular imaging of the neuropeptidergic and endocannabinoid systems in depression, this is currently hampered by the lack of available radioligands. There have been attempts to synthesise [^{11}C], [^{18}F] and [^{125}I] labelled small molecular probes to image the oxytocin receptor but these have failed preclinically due to a lack of specificity over the vasopressin 1a receptor and also due to low brain uptake (Smith et al., 2012). In addition, there has been a lack of studies evaluating radioligands for PET/SPECT imaging of the vasopressin 1a receptor reported to date. For the cannabinoid system there has been more interest and progress on the development of radioligands for imaging CB1 receptors. However, these are still to be shown to be effective for clinical imaging (Yasuno et al., 2008; Herance et al., 2011).

In this thesis, I aim to understand the influence of chronically elevated glucocorticoids on the serotonergic, peptidergic and endocannabinoid systems as regulators of the HPA axis with a view to identify potential targets within these systems for the development of PET/SPECT imaging probes The role of each in depression, and in particular stress related disorders is discussed separately below.

3.2 The serotonergic system

Serotonin (5-hydroxytryptamine, 5-HT) is a biogenic amine which was first isolated from the enteric system in 1947 (Rapport et al., 1947). In the periphery, serotonin is most abundantly found in the gastrointestinal system (90-95% of total serotonin), where it regulates enteric smooth muscle contraction

during the process of digestion (Gersohn et al., 1989). In addition, platelets also contain a high amount of serotonin, where it is responsible for vasoconstriction of blood vessels (Opacka Juffry, 2008). Interestingly, only a small portion (1-2%) of the total amount of serotonin present is found in the central nervous system (CNS). This is surprising considering the serotonin network is the most extensive and regulates a diverse range of physiological processes (more details can be found below) (Beattie and Smith, 2008).

Serotonin is synthesised from the essential amino acid $_{L}$-tryptophan which originates from diet (Lanfumey et al., 2008). However, peripherally synthesised serotonin cannot cross the blood brain barrier, so it must also be synthesised locally within the brain. Synthesis is carried out by the conversion of $_{L}$-tryptophan (which does readily enter the brain) to serotonin via a two-step process. The first step is the hydroxylation of tryptophan to the intermediate, 5-hydroxytryptophan. This occurs via the enzyme tryptophan hydroxylase, and is considered to be the rate-limiting step in serotonin synthesis. The second step involves decarboxylation of 5-hydroxytryptophan, to 5-hydroxytryptamine (serotonin or 5-HT) by the enzyme, amino acid decarboxylase. To remove serotonin from the synaptic cleft, it is transported back into the neurone and metabolised by monoamine oxidase (MAO). The major metabolite of serotonin is 5-hydroxyindoleacetic acid (5-HIAA), however, in the pineal gland, serotonin is converted to melatonin regulating circadian rhythms (Opacka-Juffry, 2008).

Centrally, serotonin production is limited to the serotonergic neurones originating in the raphé region of the brain (Mössner et al., 2004). From here, neurons project extensively throughout the brain i.e. to the hippocampus, frontal cortex and striata, making the serotonergic system the most diffuse network in

the brain (Descarries et al., 1990; McQuade and Sharp, 1997). This is shown in figure 3.1. Thus, serotonin is involved in a wide range of physiological functions e.g. thermoregulation, locomotion, sexual behaviour, appetite, cardiovascular tone and regulation of mood and emotions (Lanfumey et al., 2008). For this reason, serotonergic dysfunction is implicated in disease states such as depression, anxiety, schizophrenia, obsessive compulsive disorder, migraine and eating disorders (Lucki, 1998).

Within neurones, serotonin is stored in vesicles, which are 40 nm in diameter and concentrated at synapses, protecting the neurotransmitter from degradation by MAO (Tamir et al., 1994). Upon stimulation of the neurone by an action potential, serotonin may be released from dendrites, axons and cell bodies, to propagate nerve transmission to the contacting neurone. (De-Miguel and Trueta, 2005). As well as serotonin, the vesicles also contain a serotonin binding protein (SBP) and as the name suggests, this protein binds serotonin upon synthesis in the cytoplasm, and transports it into the vesicles for storage and subsequent release (Tamir et al., 1994).

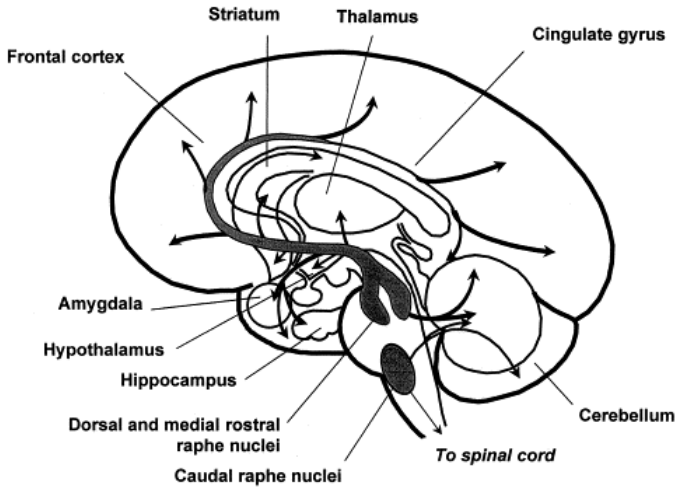


Figure 3.1. Serotoninergic innervation of the human brain (Nutt et al., 1999).

Once released, serotonin exerts its effects through the serotonin receptor, of which there are seven families (5-HT_{1-7}) and fourteen subtypes (Barnes and Sharp, 1999). In particular, the 5-HT_{1A} receptor is implicated in depression (Hoyer, 2002) along with the 5-HT_{2C} receptors (Lanfumey et al., 2008; Leventopoulos et al., 2009). However, in this thesis, due to its pivotal role in depression (as reviewed in Lanfumey et al., 2008) attention is given to the 5-HT_{1A} receptor and the influence of elevated glucocorticoids on 5-HT_{1A} receptor binding.

3.2.1 The 5-HT_{1A} receptor

The 5-HT_{1A} receptor is one of many in this class (5-HT_{1A-F}) and was identified by displacement of [^{3}H]5-HT binding by the full agonist, 8-hydroxy-di-propylaminotetralin (8-OHDPAT). Through such experiments, it was identified that a high density of 5-HT_{1A} receptors was located in the hippocampus, septum, amygdala, raphé and cortical regions of the brain (Palacios et al., 1990). The presence of 5-HT_{1A} receptors in these areas suggests a possible role for the receptor in the regulation of mood and emotion (Palacios et al., 1990).

The 5-HT_{1A} receptor exists as both presynaptic autoreceptors (somatodendritic receptors) and as postsynaptic receptors. The presynaptic autoreceptors are predominantly located on 5-HT neurone cell bodies and dendrites in the raphé region. Destruction of the serotonergic cell bodies in the raphé using the toxin 5,7-dihydroxy-tryptamine resulted in a loss of 5-HT_{1A} receptor binding in the same region confirming that the receptors are present

presynaptically. Similarly, kainic acid induced lesions of the hippocampus (which degenerates pyramidal cells and interneurons) are associated with the loss of 5-HT_{1A} receptor binding suggesting that 5-HT_{1A} receptors are located postsynaptically in the hippocampus (Palacios et al., 1990). Considering the extensive serotonergic innervation throughout the CNS, activation of 5-HT_{1A} autoreceptors influences activity in a large number of brain regions i.e. stimulation of presynaptic receptors, reduces serotonergic activity in projection areas. In addition, the 5-HT_{1A} receptor is able to interact and influence other neurotransmitter systems i.e. inhibit dopamine and glutamate release (Murphy et al., 1998).

The 5-HT_{1A} receptor subtype has a high affinity for serotonin (<10 nM) (Peroutka and Snyder, 1979). The receptor is coupled to the inhibitory G-proteins and therefore when bound by an agonist, has an overall inhibitory tone on neurotransmission. There are two G-proteins to which the receptor may bind; the G_i or the G_0. When coupled to a G_i protein (as in the raphé), production of cyclic AMP is prohibited and when coupled to G_0 (as in the hippocampus), potassium channels are prevented from opening. It is thought that differences in the G-protein coupling may explain the regional differences that exist in adaptive responses at both 5-HT_{1A} receptors (Mannoury la Cour et al., 2006). Moreover, continual stimulation by agonists is known to desensitise and internalise the presynaptic receptors in the raphé, but not the postsynaptic receptors in the hippocampus (Riad et al., 2001).

Several human polymorphisms of the 5-HT_{1A} receptor gene have been found and are linked to psychiatric disorders. One of the most extensively studied is the -1019 polymorphism which exists in some cases of depression and

anxiety. It results in an overexpression of the 5-HT_{1A} autoreceptor and hence a reduction in serotonin neurotransmission (Lemonde et al., 2003; Strobel et al., 2003). Moreover, this polymorphism is associated with poor responses to therapy e.g. drug naïve schizophrenic patients with the polymorphism exhibit a reduction in the alleviation of negative and depressive symptoms (Reynolds et al., 2006).

3.2.2 Serotonergic system and depression

There is extensive evidence for the involvement of serotonergic dysfunction in depression and related mood disorders. Although it is no longer considered to be the only player, the fact is that the contribution of the serotonin system to depression and anxiety is significant and thus cannot be ignored. Especially, since all the currently available antidepressant treatments target the serotonergic system at various levels (Den Boer et al., 2000). These are discussed further at the end of this section.

Inducing a deficit of endogenous serotonin using either reserpine or via tryptophan depletion leads to the onset of depressive symptoms and this formed the basis of the serotonin hypothesis of depression. However, both these treatment paradigms not only affect 5-HT concentrations, but also affect other neurotransmitter systems such as the dopaminergic, noradrenergic, cholinergic and glutamatergic systems. Therefore the effects of reserpine or tryptophan depletion cannot be ascribed to just the serotonin system (reviewed in Jacobsen et al., 2012). *Post mortem* analysis revealed an increase in the binding of the agonist [^{3}H]OH-DPAT in the raphé region, which the authors attributed to a reduction in serotonin activity in the brains of depressed patients (Stockmeier et

al., 1998). Whereas, low 5-HIAA levels in CSF have been associated with suicide but not necessarily depression (Nordström et al., 1994; Placidi et al., 2001). In addition, *post mortem* brain tissue from depressed patients and also those that have committed suicide express a lower concentration of 5-HT and 5-HIAA (Owen and Nemeroff, 1994).

However, there is some evidence to show that actually depression may be associated with enhanced central serotonergic activity. This is measured by an increased prolactin response to dexfenfluramine (the amphetamine analogue which causes a release of serotonin) (Strickland et al., 2002). Also, in some instances, 5-HIAA levels in CSF are reported to be higher in depressed patients suggesting increased serotonergic function (Sullivan et al., 2006).

In both humans and animals, serotonin is known to activate the HPA axis, resulting in the secretion of CRH, ACTH and glucocorticoids. This effect has been shown to be potentiated by the 5-HT_{1A} agonist, ipsapirone (Klaassen et al., 2002) and abolished by serotonin antagonists (Fuller, 1990), providing evidence for interactions between the serotonergic system and regulation of the HPA axis. Preclinical studies have reported that glucocorticoids influence the expression and function of central 5-HT_{1A} receptors. In response to chronic corticosterone administration, 5-HT_{1A} receptor mRNA density in the hippocampus, parietal cortex, prefrontal cortex and posterior cingulate is reduced, whereas, adrenalectomy resulted in an increase in 5-HT_{1A} receptor density (Mendelson and McEwen, 1991, 1992; Flugge, 1995). In addition, glucocorticoids have also been shown to affect the function of 5-HT_{1A} receptors. Low doses of corticosterone attenuated hippocampal and raphé 5-HT_{1A} receptor function, whereas high doses, enhanced 5-HT_{1A} receptor function (Joels et al.,

1991; Karten et al., 1999; Fairchild et al., 2003). Glucocorticoids have also been reported to influence the concentration of serotonin in a region dependent manner. In general, exposure to stress increases 5-HT levels in the raphé, prefrontal cortex, hippocampus and amygdala (Maswood et al., 1998; Amat et al., 2005). However decreases in 5-HT content of the amygdala and septal nuclei have also been reported after an acute stress (Kirby et al., 1995). The role of serotonin in the stress response is discussed further in chapter 5.

The most commonly prescribed antidepressants, are the selective serotonin reuptake inhibitors (SSRIs). Although they are effective treatments for depression, the response is slow and it can take several weeks of treatment before an improvement in clinical symptoms is seen. The exact mechanism of action of SSRIs is still being elucidated, but it is thought that they act by suppressing serotonin transporter-mediated 5-HT reuptake and hence increase the concentration of 5-HT in the synaptic cleft (Raap et al., 1999). This, in turn, is thought to desensitise presynaptic 5-HT_{1A} receptors, blocking their inhibitory action on the autoreceptors, and increasing serotonin release in the projection areas (Benmansour et al., 1999; Hjorth et al., 2000). The administration of pindolol, a selective 5-HT_{1A} autoreceptor antagonist used as an adjunct to SSRI treatment supports the theory of autoreceptor desensitisation as a key early event in the mechanism of action of SSRI treatment (Ballesteros and Callado, 2004). Agonists of the 5-HT_{1A} receptor have also shown to exhibit antidepressant effects. For example, the agonist gepirone desensitises presynaptic 5-HT_{1A} neurones but not postsynaptic receptors (Blier and de Montigny, 1987).

Enhancement of serotonin neurotransmission as a mechanism of antidepressant action can also be achieved by electric convulsive shock therapy

(ECS) and also by monoamine oxidase inhibitors (MAOIs) which sensitises postsynaptic 5-HT_{1A} receptors in the hippocampus (Stockmeier et al., 1992; Burnet et al., 1994) and inhibits the breakdown of 5-HT (Nemeroff, 2002) respectively. Figure 3.2 outlines therapeutic approaches to treating depression. However, irrespective of the way in which serotonin activity is enhanced, in order to achieve long-term benefit from antidepressant action, it is necessary to re-establish neurogenesis in adult brain. This is a process which is dependent on serotonin (and the 5-HT_{1A} receptor) to enhance the production of new neurons (Gould, 1999). The SSRI, fluoxetine has been shown to stimulate neurogenesis, via the 5-HT_{1A} receptors (Santarelli et al., 2003).

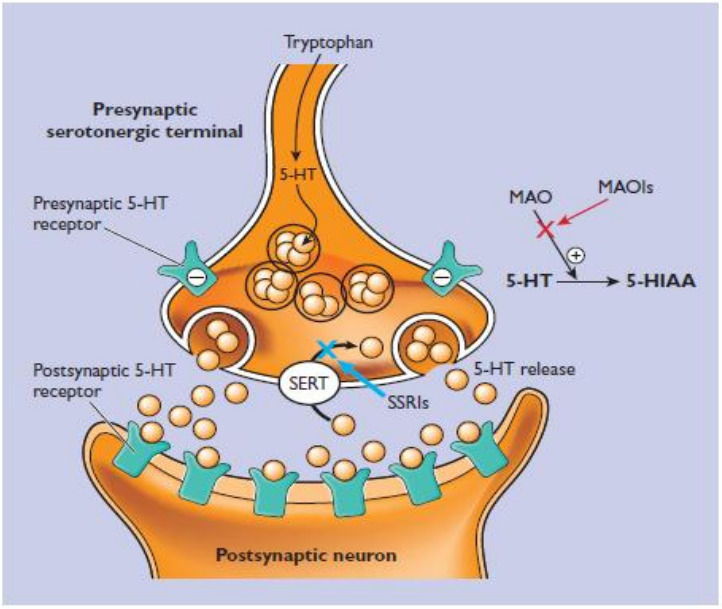


Figure 3.2. A serotonergic synapse showing mechanisms of action for common antidepressant drugs (Opacka-Juffry, 2008), where SERT = serotonin transporter, MAO = monoamine oxidase, MAOIs = monoamine oxidase inhibitors, SSRIs = selective serotonin reuptake inhibitors.

3.3 The neuropeptidergic system

Neuropeptides are molecules composed of a short chain of amino acids, they are released from the nerve terminal to act on cell surface membranes and

are then degraded i.e. they are not recycled back into the neuron (Arzumanyan et al., 1985). There are many different peptides released by the brain which participate in numerous physiological functions e.g. neuropeptide Y (NPY) regulates feeding, body weight (Patel et al., 2006) and regulates pain processes (Bjersing et al., 2012); apelin regulates drinking and fluid retention (Roberts et al., 2009); orexin regulates sleep/wake cycles (Blumber et al., 2007); galanin regulates memory (Ogren et al., 2010); kisspeptin regulates reproduction/fertility (Hameed et al 2011) and substance P regulates vomiting and nausea (Rojas et al., 2010). In addition to the neuropeptides mentioned above, there are also oxytocin and vasopressin, which are studied in this thesis. These two neuropeptides are discussed in more detail for the remainder of this section.

Centrally, oxytocin and vasopressin are synthesised in the supraoptic (SON) and PVN of the hypothalamus. The peptides are released from the hypothalamic parvocellular and magnocellular neurones. Oxytocin released from axon terminals of the parvocellular neurones, exerts local effects. Whereas oxytocin originating from the axon terminals and dendrites of magnocellular neurones will enable a more diffuse actions of oxytocin at remote distances within the brain (Baskerville and Douglas 2010; Onaka et al., 2012). In addition, there are extrahypothalamic vasopressinergic and oxytocinergic fibres which originate from the BNST and terminate in the limbic brain regions e.g. septum and hippocampus (Engelmann et al., 1996). Therefore, vasopressin and oxytocin are also released in limbic regions (Neumann and Landgraaf, 2012).

From the hypothalamus, most oxytocinergic and vasopressinergic neurones project into the posterior pituitary gland (Baskerville and Douglas 2010). From here, oxytocin and vasopressin are released into the general and

central circulation to exert their actions via the oxytocin, vasopressin 1a and 1b receptors (Neumann and Landgraaf 2012; Onaka et al 2012). This would imply that circulating peripheral concentrations of the peptide may reflect central concentrations in the brain. However this is not the case as certain stimuli may result in a rise in central neuropeptide synthesis and release without changing concentrations in the blood (Landgraaf and Neumann 2004; Neumann 2007).

There are a number of regions where the vasopressin 1a receptor is coexpressed with the oxytocin receptor, however they are located in discrete areas separate of each other. For example, oxytocin receptors are located in the rat amygdala (lateral part of central amygdaloid nucleus (CeA)) and nucleus accumbens (shell), whereas the vasopressin 1a receptor is expressed in the core of the nucleus accumbens and the medial part of the CeA. This complimentary location of the two receptors may explain the opposite effects of oxytocin and vasopressin on fear behaviour (Huber et al., 2005). Generally, oxytocin is considered to be anxiolytic and to have antidepressant like effects, whereas vasopressin is anxiogenic and has depressive properties (Neumann and Landgraaf 2012).

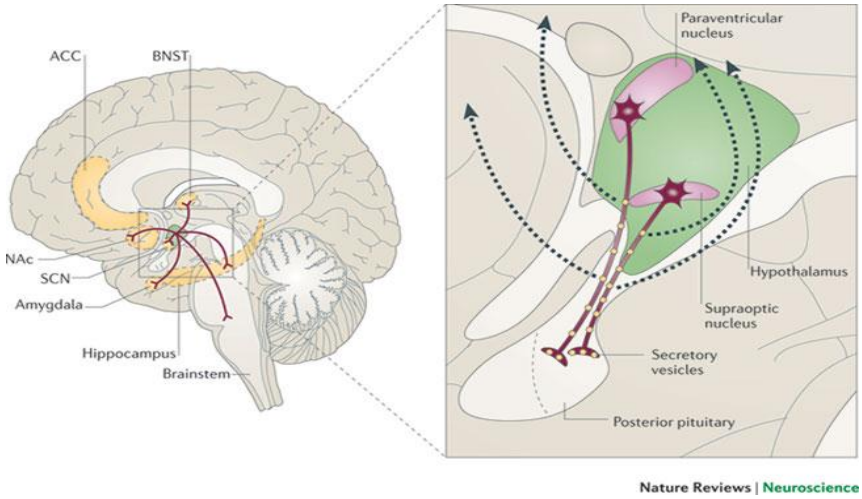


Figure 3.3. Oxytocin and vasopressin synthesis and release in brain (Meyer-Lindenberg et al., 2011)

3.3.1 The oxytocin neuromodulatory system

The hormone oxytocin participates in many physiological functions, through actions at peripheral as well as central binding sites. Peripherally, the stimulation of milk ejection (Neumann et al., 1993) and the stimulation of uterine contraction (Barberis et al., 1998) are important functions regulated by oxytocin. Centrally, oxytocin is involved in regulating emotional behaviours such as pair bonding (Insel and Shapiro 1992; Young and Wang 2004; Lim and Young 2006), maternal behaviour (Bosch et al., 2005; Lim and Young 2006), sexual arousal (Caldwell et al., 1992; Baskerville and Douglas 2010), social bonding (Popik and van Ree 1998; Ferguson et al., 2001), trust (Kosfeld et al., 2005), reducing anxiety and fear (Kirsch et al., 2005; Windle et al., 2007), processes of learning and memory (de Oliviera et al., 2007) and provides tolerance to some addictive drugs (Kovacs et al., 1998).

Oxytocin is a nonapeptide which is synthesised and released from the PVN and SON regions of the brain. It is cleaved from a pre-peptide as it is

transported down the axon to terminals in the posterior pituitary gland. From here, it is released into the blood bound to a carrier protein, neurophysin I. Oxytocin is found in high concentrations in the pituitary gland and is present in a 1:1 ratio with neurophysin (Gimpl and Farenholz 2001). Once in the general circulation, oxytocin exerts the above mentioned peripheral effects. However, oxytocin which has been released into the general circulation is not able to pass through the blood brain barrier (BBB) and enter the brain, although in some instances 0.001% has been shown to cross the BBB (Uvnäs-Moberg, 1998). Thus, oxytocin is also synthesised and released locally within the brain from axon terminals and dendrites.

Within oxytocinergic neurones, the peptide is packaged into vesicles at the axon terminals which release their contents upon the generation of an action potential (which triggers influx of Ca^{2+}) (Baskerville and Douglas 2010). On the other hand, the dendritic release of oxytocin is electrically independent and occurs in response to intracellular release of Ca^{2+} (Ludwig et al., 2002). Due to the fact that these two distinct pathways for oxytocin release exist, the circulating and central oxytocin concentrations can either be independent of each other or can occur in synergy. For example, Engelmann et al., (1999) showed that oxytocin is released by the SON without an increase in peripheral oxytocin levels, whereas rodent studies showed that suckling led to the concomitant release of oxytocin into the bloodstream as well as in the SON and PVN (Moos et al., 1989; Neumann et al., 1993).

3.3.1.1 The oxytocin receptor

The encoded oxytocin receptor is a G-protein coupled receptor which is 389 amino acid polypeptides in size and has seven transmembrane domains (Baskerville and Douglas 2010). The oxytocin receptor is structurally similar to the vasopressin receptors displaying 25% homology (Barberis et al., 1998). Oxytocin acts as a full agonist at the receptor, whereas vasopressin acts as a partial agonist (Gimpl and Farenholz 2001). Although both oxytocin and vasopressin bind to the oxytocin receptor, the latter displays a 10-fold lower affinity for the receptor. It has been reported that a 100-fold higher concentration of vasopressin is required at the oxytocin receptor to elicit the same response as oxytocin (Gimpl and Farenholz, 2001).

Upon activation, the receptor stimulates the activity of phospholipase C-ß isoforms, which leads to the generation of inositol phosphate 3 ($InsP_3$) and diacylglycerol (DAG). This in turn leads to the release of Ca^{2+} from intracellular stores and activation of PKC (Gimpl and Farenholz, 2001). These signalling pathways activate a range of cellular responses such as neuronal firing, neurotransmitter release, smooth muscle contraction, and protein phosphorylation (Baskerville and Douglas 2010). After persistent activation the oxytocin receptor becomes desensitised and internalises. In fact, 5-10 minutes of stimulation resulted in a greater than 60% internalisation of the human oxytocin receptors expressed in HEK 293 fibroblasts (Gimpl and Farenholz, 2001). These are then recycled back to the cell surface (Stoop, 2012).

Due to the involvement of the oxytocin receptor in a range of physiological processes, the receptor undergoes transient changes in its expression during development, particularly in the brain i.e. it is up- and

downregulated adaptively as the brain matures. For example, a high density of oxytocin receptors is observed in the cingulate cortex and a low density in the olfactory tubercles of the infant rodent brain but this is reversed as the brain matures to adulthood (Tribollet et al., 1992). In addition, there are marked species differences with respect to the central regional distribution of oxytocin receptors in part related to differences in function (Tribollet et al., 1992).

3.3.1.2 Oxytocin and depression

The distribution of the oxytocin receptor in brain regions associated with the regulation of mood and emotions, as well as a defined role for oxytocin in affliative behaviours suggests that this neuromodulatory system may be involved in neuropsychiatric disorders. Oxytocin is amongst the factors which regulates HPA axis activity and is in fact anxiolytic (Heinrichs and Dome, 2008). The neuropeptide transiently increases corticosterone release, resulting in activation of the glucocorticoid induced negative feedback mechanism which is then followed by a sustained suppression of the HPA axis (Peterssen et al., 1996). In this way, oxytocin exerts an inhibitory influence on the HPA axis, leading to a reduction in glucocorticoid levels (Neumann et al., 2000). Moreover, recently oxytocin has been shown to protect against glucocorticoid induced inhibition of neurogenesis and to stimulate neurite growth in the hippocampus (Leuner et al., 2012). Taken together this suggests that abnormalities in the regulation of the HPA axis by oxytocin may contribute to the HPA dysfunction seen in depression.

Preclinically, intracerebral administration of oxytocin counteracts anxiety–like behaviours such as reduced immobility during the forced swim test

and reduced vocalisation of distress in rodent models (Insel and Winslow, 1991; Windle et al., 1997). In addition, a repeated daily injection of oxytocin has been demonstrated to reduce the time spent immobile during the forced swim test (Arletti and Bertolini 1987). The anxiolytic actions of oxytocin are abolished after administration of an oxytocin receptor antagonist indicating specificity of effect to the oxytocin receptor. Preclinical studies investigating the oxytocinergic regulation of the HPA axis are discussed in more detail in chapter 6.

Although, preclinical studies have shown a clear relationship between oxytocinergic dysfunction and depression, findings from clinical studies are more contradictory. Low oxytocinergic activity has been associated with postpartum depression where first-time mothers with low infant attachment also exhibit reduced plasma oxytocin concentration (Strathearn et al., 2009). In comparison, major depression is also associated with reduced plasma oxytocin concentrations when compared to age-matched controls (Frasche et al., 1995; Scantamburlo et al., 2007). However, in contrast, other studies have shown increases in plasma (Parker et al., 2010) and salivary oxytocin concentrations (Holt-Lunstat et al., 2011) in some depressed patients. Only a small number of studies have looked at changes in CSF oxytocin levels in depression, but these findings are also inconsistent. In one study, a decrease in CSF oxytocin was found in depressed patients (Pitts et al., 1995), whereas, others have found no difference between depressed and control subjects (Demitrak and Gold 1988).

Moreover, the anxiolytic influence of oxytocin has been evaluated in humans too. Intranasal administration of oxytocin caused an increase in risk-taking behaviour (Kosfeld et al., 2005) and improved communication in both men and women during partner conflict (Ditzen et al., 2009). In addition,

performance was improved on a stress task, which involved public speaking, in the presence of social support (which reduces cortisol production and increases oxytocin release) and performance was further enhanced after oxytocin administration (Heinrichs et al., 2003). In fact, it is thought that oxytocin may be beneficial to those suffering mental illnesses that are linked to social dysfunction e.g. phobias, autism or those with a persistent fear and avoidance of social interactions (Kosfeld et al., 2005). *Post mortem* analysis of brains from depressed patients have shown a 23% increase in oxytocin-immunoreactive neurones in the PVN of the hypothalamus concomitant with HPA hyperactivity measured during life (Purba et al., 1996; Meynen et al., 2007; Wang et al., 2008). The role of oxytocin in the stress response is discussed in more detail in chapter 6.

Further evidence for a beneficial role for oxytocin in the treatment of depression comes from the observation that antidepressant therapies such as SSRIs induce the release of oxytocin; this suggests that the hormone itself may be useful in alleviating the symptoms of depression (Unväs-Moberg et al., 1999). More recently the novel oxytocin agonist, carbetocin, was shown to be comparable to imipramine in alleviating depressive behaviours in the forced swim test. These effects were abolished when an oxytocin receptor antagonist was administered suggesting carbetocin and imipramine owe their efficacy in part to actions at the oxytocin receptor (Chaviaras et al., 2010). In addition, a new drug treatment for sexual dysfunction, siladenafil, also exhibits antidepressant-like effects by evoking oxytocin release from the pituitary gland (Matsushita et al., 2012).

3.3.2 The vasopressin neuromodulatory system

Vasopressin (arginine vasopressin, AVP) is a nonapeptide hormone which is also synthesised in the magnocellular neurones of the SON and released into the general circulation as well as locally within the brain in a similar way to that described above for oxytocin. The two peptides are structurally closely related, with the only difference being that oxytocin has a leucine amino acid at position eight, whereas vasopressin contains an arginine amino acid at that position. However, it is this difference which enables each peptide to interact with their respective receptors (Barberis et al., 1998).

The physiological function of vasopressin is related to maintaining homeostatic control (Stoop, 2012). Peripherally, AVP has antidiuretic and vasoconstrictive effects. It is released in response to increased plasma osmolality, hypervolemia, hypotension and hypoglycaemia (Laszlo et al., 1991). AVP is transported along nerve fibres and released into the posterior pituitary from where it enters the general circulation (Antoni 1986). Centrally, AVP is released from parvocellular neurones of the PVN, BNST, amygdala and suprachiasmatic nucleus (SCN) (DeVries et al., 1985). The neuropeptide is stored in large dense-core vesicles (LDCV) which are distributed in the soma, in dendrites, and in axonal varicosities (Stoop, 2012). The release of the peptide from these vesicles is dependent on a rise in intracellular Ca^{2+} concentration (Stoop, 2012).

In the brain, AVP mediates emotions such as aggression, fear, stress, anxiety, regulation of the circadian rhythm (Hofman and Swaab 1994) and is also implicated in memory and learning (Caldwell et al., 2008). It is released

with circadian rhythmicity into the CSF, and peaks during the middle of the light phase (Caldwell et al., 2008).

3.3.2.1 The vasopressin receptors

Vasopressin exerts its effects through plasma membrane bound receptors. There are two subtypes of the vasopressin receptor; the V1 and V2 receptors. The V1 receptors are further subdivided into vasopressin 1a (V1aR) and vasopressin 1b (V1bR) receptors (Barberis et al., 1992). All three subtypes are found in the CNS, but the density of V1 receptors is greater than V2 receptors (Tribollet 1992). The receptors are G-protein coupled with seven transmembrane domains. The V1a and V1b receptor subtypes are coupled to the Gαq/11 protein which activates PLC, but the V2 receptor is coupled to the G_s protein and activates cyclic AMP (Caldwell et al., 2008). The V2 receptor is primarily found in the kidney and collecting ducts (Narayen and Mandel, 2012) and will not be considered further here. The focus of this study is given to the vasopressin 1a receptor subtype and is therefore discussed in more detail below.

The V1a receptor is predominately distributed in the brain, but can also be found in peripheral organs e.g. kidney, liver, spleen, platelets and smooth muscle. In the brain, autoradiography and in situ hybridization studies have revealed high densities of the V1a receptor in the septum, hippocampus, amygdala, BNST, hypothalamus, substantia nigra, dorsal raphé and superior colliculi (Johnson et al., 1993). The vasopressin 1a receptor can signal independently of PLCβ, PKC or intracellular calcium concentration. AVP will affect neuronal excitability through the opening of nonspecific cationic channels or by the closing of potassium channels. Similarly to the oxytocin receptor,

activation of the vasopressin 1a receptor leads to internalisation of the receptor (Innamorati et al., 1998).

3.3.2.2 Vasopressin and depression

The distribution of vasopressin receptors as well as the role of vasopressin in the regulation of normal memory processes and circadian rhythms governing sleep patterns, suggests a role for AVP in depression. Particularly, as both these functions are disrupted in depression (Scott and Dinan, 2002). In contrast to oxytocin, vasopressin is anxiogenic and aggression promoting via activation of the HPA axis, particularly during chronic stress (Aguilera et al., 2007). In fact, AVP transcription is enhanced by glucocorticoids during stress (Kovacs et al., 2000). The neuropeptide is a weak secretagogue of ACTH, but acts synergistically with CRF to release ACTH when the HPA axis is activated (Carrasco and Van der Kar, 2003).

Preclinical studies have shown exposure to stress induces AVP release in the PVN, SON, septal nuclei and amygdala (Wotjak et al., 1998). Brattleboro rats have a genetic disposition towards vasopressin deficiency which prevents activation of the HPA axis and therefore, this strain of rat is protected from glucocorticoid induced neurological damage providing evidence for a role of vasopressin in neurological disorders (Fodor et al., 2012). In fact, Brattleboro rats show less immobility in the forced swim test suggesting that this antidepressive behaviour is partly explained by the lowered vasopressin activity (Mlynarik et al., 2007). Conversely, a high anxiety-bred strain of rat showed hyper anxiety associated with overexpression of vasopressin neurones in the PVN, which was normalised by the antidepressant, paroxetine (Keck et al.,

2003). Furthermore, the vasopressin V1a receptor is implicated in mediating the anxiogenic effects of AVP, where V1aR overexpression is associated with increased anxiety (Landgraaf et al., 1995) and vasopressin 1a receptor knockout mice display less anxiety (Bielsky et al., 2004). The role of vasopressin in the stress response is discussed further in chapter 6.

There have been fewer clinical investigations looking at the relationship between vasopressin and depression than those reported for oxytocin. However, increased levels of circulating AVP have been found in depressed patients (van Londen et al., 1997; Meyen et al., 2006; Bao and Swaab, 2010). In addition, plasma AVP levels were further increased in patients who have attempted to commit suicide when compared to those depressed patients that were not suicidal (Inder et al., 1997; Merali et al., 2006). Centrally, vasopressinergic neurones in the hypothalamic PVN and SON are activated in depression, contributing to an increase in the release of ACTH from the pituitary gland. Not only are they activated, but the number of vasopressinergic neurones and also vasopressin 1a receptor mRNA was increased in depressed patients (Wang et al., 2008). In addition, a *post mortem* study showed increased activation of vasopressin neurons in the paraventricular nucleus of depressed subjects (Purba et al., 1996).

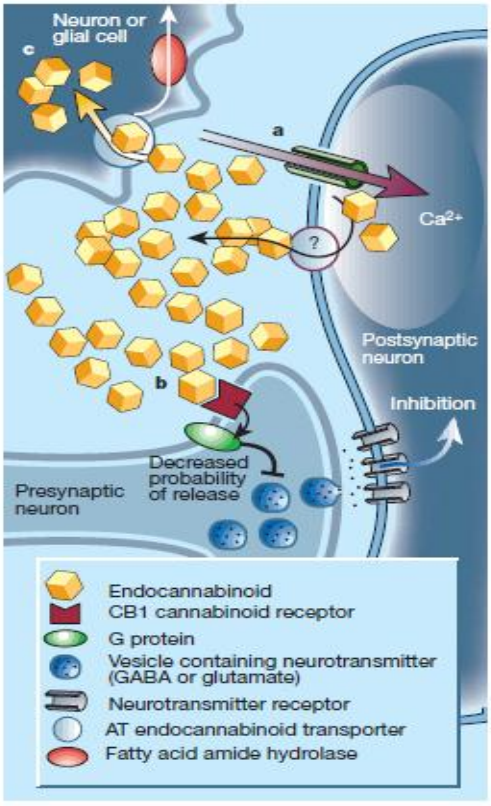
Currently there are no available antidepressants which target the vasopressin system. However, the vasopressin 1a receptor, antagonist d(Ch2)5Tyr(Me)AVP, when administered into the mediolateral septum and the amygdala has been shown to have antidepressant-like effects (Ebner et al., 1999, 2002).

3.4 The endocannabinoid system

Endocannabinoids are synthesised from polyunsaturated fatty acids. They are synthesised and released on demand in response to an increase in intracellular calcium (Di Marzo et al., 2005). The first endogenous cannabinoids to be discovered were anandamide (*N*-arachidonoyl ethanolamine) and 2-arachidonoylglycerol (2-AG) and these are the most widely studied.

Endocannabinoids have been shown to be synthesised peripherally (Sugiura et al., 1996) as well as in the brain (Devane and Axelrod, 1994). Centrally, these lipid mediators are involved in processes that regulate eating, anxiety, memory, learning, reproduction, metabolism, growth as well as being involved in neuroinflammation and neurodegeneration (Skaper and Di Marzo, 2012). Multiple pathways exist for the synthesis of anandamide and 2-AG. Anandamide is produced from the precursor N-acyletholamine phospholipid. The most common pathway for synthesis of 2-AG is from the hydrolysis of phosphotidylinositol 4,5 bisphosphate into the intermediate secondary messenger diacylglycerol (DAG). This is then converted to 2-AG by the enzyme diacylglycerol lipase (DAGL) (Skaper and Di Marzo, 2012). Endocannabinoids are removed from the synapse via uptake into cells and enzymatically degraded by several enzymes. The major metabolite formed from degradation of both anandamide and 2-AG is arachidonic acid, but the enzymes involved are different. Anandamide is hydrolysed by cyclooxygenase 1 (COX1), fatty acid amide hydrolase 1 and 2 (FAAH1, FAAH2) and *N*-acyletholamine hydrolysing acid amidase (NAAA). Whereas, 2-AG is metabolised by cyclooxygenase 2 (COX2), monoacylglycerol lipase (MAGL) or αβ-hydrolase 6 and 12 (ABHD6, ABHD12) (Muccioli, 2010).

Endocannabinoids are retrograde messengers; when released from the postsynaptic neurones they act at the presynaptic neurone to terminate the signal (Christie and Vaughan, 2001). They are highly lipophilic allowing them to readily cross most cell membranes although, specific cannabinoid transporters also exist that aid in their transport across certain membranes (Hilliard and Jarrahian 2003; Alexander and Cravatt 2006). One such transporter is present at hippocampal neurones and glial cells. It facilitates endocannabinoid signalling by transporting anandamide and 2-AG from the synapse (Wilson and Nicoll, 2001).



(a) An action potential results in the influx of Ca2+, which causes the synthesis and release of endocannabinoids .

(b) These act at CB1 receptors on the presynaptic neurone to inhibit the release of neurotransmitter

(c) The endocannabinoids are removed from the synapse through enzymatic degradation in glial or neurone cells

Figure 3.4. Retrograde endocannabinoid signalling (Christie and Vaughan, 2001).

3.4.1 The endocannabinoid receptors

Endocannabinoids exert their effects through the CB1 and CB2 receptors which differ in their distribution and signalling mechanism, and therefore regulate different physiological functions. The greatest density of the CB1 receptor is found in the brain, and much lower concentrations of the receptor are found in neurones within the heart, vas deferens and small intestine (Mackie 2005; Howlett 2000). On the other hand, the CB2 receptor forms part of the immune system and is primarily found in peripheral tissues e.g. spleen and tonsils. Until recently, it was thought that the CB2 receptor was only found in peripheral tissues, where it is responsible for mediating the immune response through modulating cytokine release. However CB2 receptors are also located in the CNS, albeit at lower densities than the CB1 receptor (Roche and Finn 2010). Centrally, CB2 receptors are expressed on neurones and glial cells in the cerebral cortex, hippocampus, striatum, amygdala, thalamic nuclei, periaqueductal grey, cerebellum (Ashton et al., 2006; Gong et al., 2006; Onaivi et al., 2006; Brusco et al., 2008). These receptors are involved in neuroinflammatory and neurodegenerative disorders e.g. multiple sclerosis, Alzheimer's disease, Huntingdon's disease, Parkinson's disease and traumatic brain injury (Pazos et al., 2004; Molina-Holgado et al., 2005; Benito et al., 2008). CB2 receptors are also present in the spine and modulate nociception processes (Sang et al., 2006). However, this thesis investigates the influence of chronic exposure to corticosterone on the CB1 receptor and therefore the CB1 receptor will be the focus for the remainder of this section.

In the brain, the CB1 receptor is regionally distributed with a high density of CB1 receptors found in basal ganglia and cerebellum, where they are

responsible for movement. A high density is also found in the hippocampus and cerebral cortex, where the CB1 receptor participates in processes associated with short term and long term plasticity i.e. memory processing, cognition and reward processes (Skaper and Di Marzo, 2012). In addition, the CB1 receptor also contributes to pain modulation and therefore is densely localised in the spinal cord and periaqueductal gray (Pertwee, 2005). The receptor is primarily located at the presynaptic terminal and its activation by retrograde endocannabinoids inhibits the release of GABA and glutamate (Rea et al., 2007).

Signalling is thought to be mediated through $G_{i/o}$ proteins, negatively coupling the CB1 receptor to adenylyl cyclase and positively coupling then to mitogen-activated protein kinase (Howlett et al., 2002). In addition, the CB1 receptor is also positively coupled to potassium ion channels and negatively to calcium channels (Howlett 2000). It has been demonstrated that the CB1 receptor can activate adenylyl cyclase through coupling to G_s proteins, a mechanism that may allow for cross-talk between CB1 receptors and other non-CB1 receptor G-protein receptors such as the serotonergic receptors (Glass and Felder 1997; Calandra et al., 1999; Breivogel and Childers 2000; Jarrahian et al., 2004). The CB1 receptor is particularly important during brain development, maturation and connectivity via regulation of neural progenitor cell proliferation, pyramidal specification and axonal growth (Skaper and Di Marzo, 2012). It is well known that the CB1 receptor is involved in the regulation of food intake and energy expenditure, and CB1 receptor antagonists/inverse agonists have been developed as potential anti-obesity agents. However, repeated administration of these agents has been shown to be associated with anxiety and depression (Bermudez-Silva et al., 2010).

3.4.2 Endocannabinoids and depression

The CB1 receptor is found in the brain regions responsible for anxiety, aversive and defensive behaviours such as the prefrontal cortex, hippocampus, hypothalamus, amygdala and periaqueductal gray (Herkenham et al., 1991; Tsou et al., 1998). Although, the role of the endocannabinoid system in neurological disorders is still emerging, endocannabinoids are known activators of the HPA axis and thus increase secretion of glucocorticoid (Weidenfeld et al., 1994; Wenger et al., 1997). Preclinically, exposure to various stressors consistently results in secretion of endocannabinoids e.g., food deprivation increases endocannabinoid levels in the hypothalamus and limbic forebrain regions (Kirkham et al., 2002), retrieval of aversive memories increases endocannabinoid levels in the amygdala (Mariscano et al., 2002), pain increases endocannabinoid synthesis in the periaqueductal gray (Walker et al., 1999) and repeated restraint stress elevates endocannabinoid secretion in the amygdala (Patel et al., 2009). However, in contrast, chronic unpredictable stress decreases secretion of 2-AG in the hippocampus (Hill et al., 2005) suggesting stressor specific changes in endocannabinoid concentration. Furthermore, exposure to stress downregulates the CB1 receptor in the hypothalamus (Wamsteeker et al., 2010) and the hippocampus (Hill et al., 2005; Reich et al., 2009; Hill et al., 2009).

Interestingly, animal studies have shown that endocannabinoids can exert anxiogenic as well as anxiolytic effects; where low doses are anxiolytic (Berrendero and Maldonado 2002) and high doses are shown to be anxiogenic (Arevalo et al., 2001; Genn et al., 2004). These effects are also reflected with cannabis use in humans, where recreational use of cannabis is anxiolytic but

chronic use can lead to anxiety and panic attacks (Thomas, 1996; Tournier et al., 2003). Moreover, inhibition of FAAH has been shown to cause an elevation of anandamide in the brain suppressing anxiety–like behaviour in rats and mice (Kathuria et al., 2003; Fegley et al., 2005; Bortolato et al., 2007; Moreira et al., 2008). This effect is abolished in the presence of the CB1 receptor specific antagonist, rimonabant, suggesting specificity of the anxiolytic function of anandamide to the CB1 receptor (Moise et al., 2008). Similarly, CB1 receptor knockout mice also exhibit increased anxiety and depressive-like behaviours (Haller et al., 2002; Maccarone et al., 2002). In addition, CB1 receptor gene variants showed increased susceptibility to stress-related disorders and depression (Juhasz et al., 2009).

Clinically, an increase in CB1 receptor density has been reported in the prefrontal cortex of depressed and suicide victims (Hungard et al., 2004; Vinod and Hungund 2006). Serum levels of anandamide and 2-AG are reduced in depressed patients, and correlates with the duration of depressive episode (Hill et al., 2009).

There still remains much to be understood regarding the potential therapeutic value of targeting the endocannabinoid system for treatment of depression and/or stress related disorders. At present only 3 drugs that activate the CB1 and CB2 receptors have been approved and these are for symptomatic management of cancer i.e. amelioration of chemotherapy induced nausea and vomiting and stimulation of appetite. However, an analysis of the pharma pipeline has revealed candidates in development for anxiety and depression too (Thomson Reuters, Integrity ™, 2012). Endocannabinoid ligands, FAAH inhibitors and anandamide reuptake inhibitors are all being investigated as

potential treatment targets for depression. Preclinical studies evaluating these novel drug candidates have reported antidepressive behaviour during the forced swim test, mediated primarily through the CB1 receptor (reviewed in Roche and Finn, 2010). More details on the endocannabinoid in stress related depression can be found in chapter 7.

In the next chapter, I describe the techniques and outline detailed protocols that are used in this thesis to investigate each of the above mentioned neurotransmitter systems in the aetiology of stress related mood disorders.

Chapter 4

General Methodology

Many different experimental approaches are used to induce a state of depression or anxiety in laboratory animals. These allow the function of the HPA axis and its involvement in the aetiology of stress-induced depression to be studied. Such methods have included exogenous administration of glucocorticoids or exposure to a psychological or physical stressor that activates the endogenous stress mechanism. The effects of such approaches can then be assessed using either behavioural tests or through measuring changes in physiological and biochemical markers. In this chapter, I outline the techniques which have been used as part of this thesis. I also give details of the materials and methods used for studies conducted as part of this investigation. In addition, I outline a feasibility study to determine the optimum method for corticosterone administration to rodents.

4.1 Introduction

In order to assess the effects of glucocorticoids in depression, various techniques can be used to increase the level of the stress hormone, corticosterone, in a rat. These can be grouped into physical stressors such as chronic social (Mckittrick et al., 1995, 2000; Bodnoff et al., 1995; chronic restraint (McLaughlin et al., 2007, Magarinos and McEwen 1995, Watanabe et al., 1992, Gregus et al., 2005) and chronic unpredictable stress (Lopez et al., 1998) or experimental stressors involving the direct administration of corticosterone via subcutaneous pellet implantation (Akana et al., 1992; Chalmers et al., 1993; Meijer et al., 1997; Karten et al., 1999; Bodnoff et al., 1995; Bisagno et al., 2000; Bush et al., 2003), subcutaneous injections (Watanabe et al., 1992; Karten et al., 1999; Czyrak et al., 2002; Gregus et al., 2005; Jacobsen and Mork 2006; Zahorodna et al., 2006) or via drinking water (Magarinos et al., 1998; Fairchild et al., 2003; Donner et al., 2012).

The addition of corticosterone to drinking water is a relatively new and non-invasive method for the administration of corticosterone throughout the diurnal rhythm (Magarinos et al., 1998). Both cases of stressor (physical and experimental) have been shown to cause neurochemical and behavioural changes in the rat which correlate to stress-induced depression as seen in humans. These include changes in physiological parameters such as an increase in plasma corticosterone levels and attenuated weight gain (referenced as above). In this thesis, exogenous corticosterone is administered to rats. The different methods reported to achieve this in laboratory animals are briefly outlined below.

4.2 Study design used in this thesis

This thesis outlines experimental studies which investigate the effects of chronic exposure to exogenous corticosterone on receptor binding at central 5-HT_{1A}, oxytocin, vasopressin 1a and CB1 receptors in adult rats. In addition, I attempt to relate changes in receptor binding to changes in the concentration of endogenous ligand. The study was designed as an experiment with between group comparisons. Age-matched male Wistar rats were randomly assigned to one of two groups, either a group which was exposed to high doses of the stress hormone, corticosterone or to a control group which received vehicle (0.8% ethanol). The independent variable was the treatment (corticosterone or vehicle) whilst the dependent variables were central receptor binding and concentration of neurotransmitter.

4.2.1 Ethical considerations

The experiments were conducted across both the GE Healthcare (Hammersmith Imanet, Hammersmith Hospital, London) site and Roehampton University. All animal treatments and *in vivo* studies were approved under The Animal (Scientific Procedures) Act, 1986. The study was approved by both the Imperial College, Ethical Review Process (ERP) and Roehampton University Ethics Boards (Appendix A). The work was carried out under the Home Office project licence (PPL 70/6744) in the Imperial College London designated facilities at Hammersmith Hospital.

The number of animals used in this thesis was justified by previous work carried out at GE Healthcare and Roehampton University, which established the minimum group size required to observe statistically significant changes in the

variables of interest assuming biological effects of 15% and above. Guidance was also sought from The Home Office with regard to the principle of '3Rs' (Reduction, Refinement and Replacement). This ensured that the study design and methodology utilised a minimal number of animals and that alternatives to the use of animals had been considered.

The post mortem brain tissues were harvested and frozen at GE Healthcare and transported to the University of Roehampton, Department of Life Sciences for processing.

In the next section, I outline a feasibility study to determine the optimum dosing regime to be used for further studies presented in this thesis (i.e. those presented in chapters 5, 6 and 7). Then after that I outline the theory, materials and methods used for experiments as part of this study.

4.3 Feasibility study

In order to study the influence of chronic exposure to glucocorticoids on receptor binding, it was decided to administer exogenous corticosterone at a dose that would result in a flattening of the diurnal rhythm of corticosterone release, and also in an attempt to minimise inter-individual differences associated with psychosocial stressors. In this section, I describe a feasibility study which was conducted to determine the route of exogenous corticosterone administration for further studies.

4.3.1 Feasibility study - Introduction

As described above, a feasibility study was designed to identify the optimum corticosterone treatment paradigm. The endpoints i.e. A success criterion was set as suppression of the HPA axis (assessed by thymus and adrenal gland atrophy) without breaching the weight loss severity limits imposed by the Home Office project licence. A reduction in body weight was expected after corticosterone treatment; however a loss greater than 20% would necessitate termination of the animal and exclusion from the study. Animals were administered CORT via daily subcutaneous injections (40 mg/kg, Gregus et al., 2005) or via addition to their drinking water (400 µg/mL, Magarinos et al., 1998) for 21 days. Body weight measurements were taken throughout the treatment period and at sacrifice adrenal glands and thymus glands were dissected and weighed.

4.3.1.1 Exposure to corticosterone by subcutaneous injections

An effective method for increasing corticosterone concentration in animals is through subcutaneous injections. Corticosterone is virtually water-insoluble but suspensions in oil, tween and saline have shown to elevate plasma CORT levels. A 10 mg/kg suspension dose of CORT resulted in an occupancy of the glucocorticoid receptor for most of the day (Karten et al., 1999; Watanabe et al., 1992). In addition, a positive correlation was found to exist between CORT dose and depression like behaviour, with a 40 mg/kg dose producing the most significant increase in depression like behaviour during the social interaction test, open field and forced swim tests (Johnson et al., 2006; Gregus et al., 2005).

4.3.1.2 Exposure to corticosterone in drinking water

Addition of corticosterone to drinking water is a relatively new and non-invasive method for the administration of corticosterone, with the advantage that the hormone is accessible throughout the diurnal rhythm (Magarinos et al., 1998). Corticosterone, initially dissolved in a small amount of ethanol (0.8 % of total volume) whilst stirring over gentle heat, is simply diluted with tap water and made available to the animals (Rees et al., 2004). The amount of corticosterone ingested can be calculated based on an assumption that each rat consumed an equal volume of water (Fairchild et al., 2003). This approach has been used by Magarinos et al., 1998, who report a decrease in adrenal, spleen, and thymus gland weights, as well as an attenuation in weight gain after treatment with 400 µg/mL of CORT for 21 days. They also report atrophy of the hippocampal brain region. It is thought that such changes may contribute to the loss of hippocampal volume, which is typically observed in depressed patients and provides an explanation for the reduction in learning and memory behaviours associated with depression (Manji et al., 2003).

4.3.2 Feasibility study – Methodology

Young adult (2 months old) male Wistar rats (Harlan Olac, U.K.) were randomly assigned to either a corticosterone (CORT) or vehicle treated group (mean initial body weight = 220.7g ± 6.5g (mean ± SD); n=12 in each group). The CORT treated group was further divided so that half were administered daily subcutaneous injections of CORT, whereas the other half were administered CORT via addition to drinking water. Each CORT group had a parallel control group that received vehicle. The groups of animals were not

counter balanced for weight, but were randomly assigned to each treatment. The segregation of animals is schematically described in figure 5.1. For identification purposes each rat was assigned a code as this is also described in figure 5.1. Animals were kept on a 12 hour light:dark cycle (lights on at 7.30am and off at 7.30pm) and at ambient temperature (19-21^{0}C). They were housed in cages of three with access to food (Charles River, UK) and water *ad libitum.* Animals were acclimatised for 7 days before the study commenced, during which time the animals were regularly handled.

For administration by subcutaneous injections, corticosterone (Sigma, Poole, Dorset, UK) was given at a dose of 40 mg/kg suspended in physiological saline. Approximately 0.5 mL of Tween (Sigma, Poole, Dorset, UK) was added to stabilise the suspension. Each day, animals were weighed prior to receiving either corticosterone or vehicle in a volume of 1 mL/kg into the subcutaneous tissue surrounding the neck. All animals were treated for 21 days with dosing carried out before 11am each day. A similar treatment paradigm has been used previously by Gregus et al., 2005 who demonstrated that corticosterone treated rats showed depression like behaviour in the forced swim test.

For administration via drinking water, corticosterone (Sigma, Poole, Dorset, UK) was dissolved in ethanol (Rees et al., 2004) and then diluted with tap water to give a final concentration of 400 µg/mL. A parallel group of rats had an equivalent amount of ethanol added to their drinking water (0.8% of total volume). Animals were weighed every day for the duration of the treatment period. Water bottles were also weighed to measure the volume of water consumed. Corticosterone and vehicle drink solutions were replaced every second day with a freshly prepared solution. Water bottles were wrapped in

aluminium foil to protect from light as the stability of corticosterone in light was not known. This treatment was based on that described in Magarinos et al. (1998) and more recently Donner et al. (2012).

Formal behavioural testing was beyond the scope of the studies presented in this thesis, however, each day an informal observation was made on the general appearance of the animals and for any signs of distress.

At the end of the treatment period, animals were sacrificed after a 24 hour hormone free period (van Gemert et al., 2006). This was included to negate any effects of an acute dose of corticosterone on the variables measured. The animals decapitated whilst very lightly anaesthetised i.e. an anaesthetic box was filled with isoflurane and nitrous oxide/oxygen gaseous anaesthetic for 3 mins, the rat was placed inside the chamber for 1 min before removal and decapitation. Animals were sacrificed in a random manner across treatment groups and during the first part of the light phase i.e. before 11am. Brain tissue generated from the feasibility study was used for training purposes (cryosectioning or regional brain microdissection). Whole brains were either immediately frozen at -40^0C in isopentane or hand-dissected into the following regions: prefrontal cortex; striatum; hypothalamus; frontal cortex; amygdala; dorsal hippocampus; ventral hippocampus; and periaqueductal gray and snap frozen in isopentane (-40^0C) on dry ice.

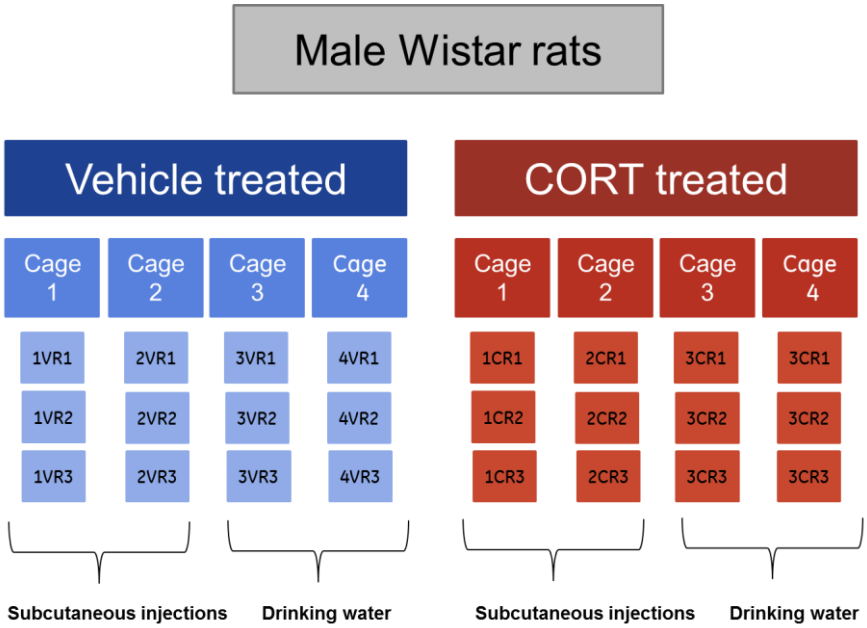


Figure 4.1 Experimental design and animal identification. Rats were randomly assigned to either a vehicle (V) or CORT (C) treated group (n=12 in each treatment group) divided between 4 cages. Animals were further divided into a subgroup that received CORT via subcutaneous injection and another that received CORT via addition to their drinking water (n=6 per subgroup and per treatment group). Each rat within the cage was identified by the following code 'cage number, treatment group, animal number' i.e. 1VR1 or 1CR1. Animals in cages 1 and 2 were assigned to the subcutaneous injection group and animals in cages 3 and 4 were assigned to the drinking water group.

4.3.1.3 Feasibility study – Results

All data presented here is expressed as mean ± SD in the text and table, whereas graphically, they are expresses as mean ± SEM. In addition, data were tested for and passed the Kolmogorov-Smirnov test for normality.

At sacrifice, the thymus and adrenal glands (left and right side combined) were dissected and weighed. Organ weights are expressed as mg/100 g body weight and are shown in table 4.1. Student's two-tailed t-test (unpaired) was used to test for effects of CORT treatment on organ weights. Statistical analysis is shown in appendix B. Each treatment paradigm was tested separately i.e.

vehicle vs CORT in subcutaneous injection treated group and vehicle vs CORT in drinking water group. Both glands were significantly reduced in weight after CORT treatment when compared to vehicle treated animals irrespective of the method for CORT administration. Daily subcutaneous injection of CORT for 21 days resulted in a decrease in adrenal gland weight from 10.38 ± 1.5 mg/100g body weight in the vehicle treated group to 3.68 ± 1.6 mg/100g body weight in CORT-treated animals representing a 64.4% reduction (P<0.0001). Thymus gland weights were also reduced from 172.90 ± 15.8 mg/100g body weight in the vehicle treated group to 85.71 ± 22.3 mg/100g body weight in CORT-treated animals representing a 50.4% reduction in weight (P<0.0001). When CORT was administered via drinking water for 21 days, adrenal glands were reduced in weight from 19.27 ± 3.4 mg/100g body weight to 6.86 ± 0.8 mg/100g body weight, representing a 64.2% reduction (P<0.0001). Thymus gland weights in this group were also reduced from 178.70 ± 18.8 mg/100g body weight to 91.29 ± 40.1 mg/100g body weight, representing a 48.9% reduction (P=0.0007).

Table 4.1. Effect of chronic exposure to CORT on thymus and adrenal gland weights.

Organ	**Vehicle group (organ weight (mg) /body weight (g))** Subcutaneous injection	**CORT group (organ weight (mg) /body weight (g))** Subcutaneous injection	**% change**	**Vehicle group (organ weight (mg) /body weight (g))** Drinking water	**CORT group (organ weight (mg) /body weight (g))** Drinking water	**% change**
Adrenal glands	10.38 ± 1.5	3.68 ± 1.6 ***	64.4	19.27 ± 3.4	6.86 ± 0.8***	64.4
Thymus	172.90 ± 15.8	85.71 ± 22.3 ***	50.4	178.70 ± 18.8	91.29 ± 40.1 ***	48.9

Table 4.1 At sacrifice, adrenal and thymus glands were dissected and weighed. Tissue weight (mg) was normalised to the individual body weight of animal (g) (n=6 per treatment group). Data are expressed as mean ± SD. Statistical analysis was via Student's two tailed t-test (unpaired) where, ***P<0.001.

Weight gain was measured throughout the treatment period to ascertain the tolerability of the CORT treatment. Vehicle treated animals in both the subcutaneous injection and drinking water groups gained weight in the expected manner. When CORT was administered by subcutaneous injection, significant effects of treatment ($F(1,210) = 1223$, $P = <0.0001$) and duration of treatment ($F(20, 210) = 20.84$, $P = <0.0001$) on weight gain were observed. The interaction was also significant ($F(20,210) = 24.64$, $P = <0.0001$). When CORT was administered via drinking water, weight gain was also attenuated with significant treatment effects ($F(1,210) = 1059$, $P = <0.0001$) and duration of treatment effects ($F(20,210) = 26.06$, $P = <0.0001$). The interaction was also significant ($F(20,210) = 17.80$, $P = <0.0001$). Data are shown in figure4.2 (A) and (B). Statistical analysis is shown in appendix B. Further analysis between treatment groups at individual time points using two-tailed Student's t-test (unpaired) with Bonferroni correction with a significance level set at $P = 0.05$, revealed that weight gain was attenuated in CORT treated animals for both administration paradigms. In the subcutaneous injection group, vehicle treated rats steadily gained weight with an increase from 211g ± 7g to 291g ± 19g, representing a 38% increase in body weight over the duration of the study. However, the mean weight of CORT-treated animals changed from 213g ± 6g to 214g ± 17g representing only a 0.5 % increase in body weight over the same period. In the drinking water group, vehicle treated rats also steadily gained weight with an increase from 232g ± 9g to 324g ± 16g, representing a 40 % increase in body weight over the duration of the study. However, the mean weight gain of CORT-treated animals increased from 226g ± 10g to 236g ± 20g representing only a 4%

increase in body weight over the same period. Thus, treatment with CORT for 21 days via daily subcutaneous injections or via addition to drinking water significantly attenuated weight gain in male Wistar rats.

Furthermore, a two way ANOVA with bonferroni correction was used to test for statistical differences between the two different treatment paradigms. There was no effect of administration method on the rate of weight gain in the treated groups.

Analysis of water consumption in the group treated with corticosterone via addition to drinking water showed that CORT treated animals ingested an average of 52.2 mg/kg of CORT per day. This was based on an average water consumption of 30 mLs of water per animal per day (assuming that each animal consumed an equal amount of water).

It is worth noting that the animals receiving daily CORT injections displayed a greater degree of distress, assessed by their general appearance i.e. hunched posture, lack of grooming and rearing behaviour in the home cage, than those administered CORT in drinking water.

4.3.1.4 Feasibility study – Discussion

The feasibility study was designed to determine a suitable dosing regimen for subsequent studies. Both CORT treatment methods resulted in atrophy of the adrenal and thymus glands indicative of suppression of the endogenous HPA axis, and this was consistent with previous studies looking at the effects of exogenous corticosterone administration (Magarinos et al 1998; Karten et al., 1999; Gregus et al., 2005; Donner et al., 2012). In addition, both CORT treatment paradigms resulted in a reduction of weight gain similar to that

previously reported (Magarinos et al., 1998; Gregus et al., 2005; Donner et al., 2012). Although there was no significant statistical difference in the rate of weight gain between the group receiving CORT via subcutaneous injection and that receiving CORT via drinking water, the administration of CORT via daily subcutaneous injection was considered to be a more severe treatment due to the more pronounced attenuation in weight gain. In addition, these animals displayed a general appearance which was indicative of distress i.e. hunched posture, lack of grooming, discolouration of fur and resistance to handling. When CORT was administered in drinking water, weight gain was also attenuated in the CORT treated group however, these animals did not show the above mentioned signs of distress and were easier to handle. In addition, the vehicle treated rats showed a normal pattern of weight gain during the treatment period.

In conclusion, it was decided to proceed with the drinking water route of CORT administration for further experimental studies. This was decided based on the above findings that CORT administered via drinking water resulted in suppression of the HPA axis but with less distress caused to the animals.

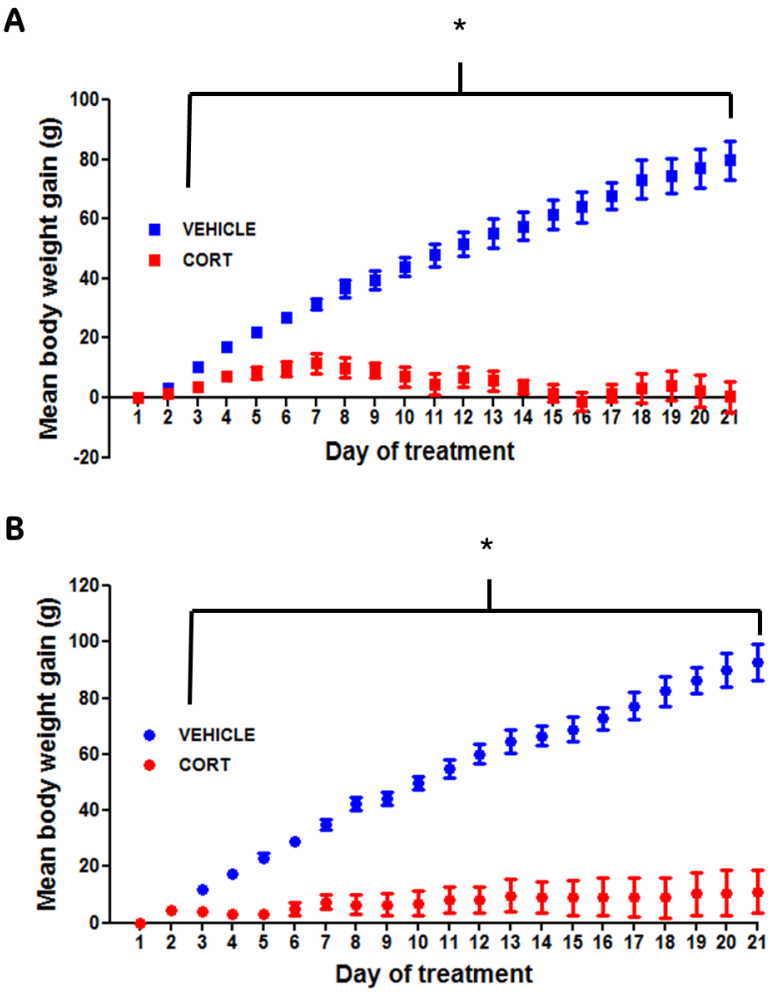


Figure 4.2. Weight gain of vehicle and CORT treated animals. Each animal was weighed every day and weight gain in grams from day 0 is plotted. Figure 4.2 (A) represents the subcutaneous injection group and (B) the drinking water group. Data are expressed as mean ± SEM of n=6 per treatment group. Statistical analysis was carried out using 2-way ANOVA for effects of treatment and the duration of treatment with a Bonferroni post hoc test for multiple comparisons at a significance level of 0.05. A statistically significant effect is denoted by *.

4.4 Techniques used in the present thesis

The theoretical background for each of the techniques utilised in this thesis are given followed by detailed materials and methods used for each experiment.

Wistar rats were used for all experimental studies described in this thesis. They are an outbred strain commonly used in stress biology research due to their susceptibility to glucocorticoid induced pathology (Karten et al., 1999; Leventopoulos et al., 2009). Stress resistant and more stress susceptible strains also exist as well as strains specifically bred for high or low anxiety states (Keck et al., 2005). Rats are social animals and prefer to be group housed with a 12 hour light period. Being nocturnal animals, they will primarily feed during the dark phase. On average, rats eat 5g of feed per 100g bodyweight daily and drink 10mls of water per 100g body weight daily. They have a naturally exploratory nature and will take interest in their environment. Failure to do so indicates poor health. Other indicators of distress and/or pain are increased vocalisations, aggressive behaviour and resistance to handling or struggling (Wolfensohn and Lloyd 2nd Edn).

4.4.1 *In vivo* corticosterone administration for subsequent studies

This section describes the corticosterone administration protocol which was used for the studies outlined in experimental chapters 5, 6 and 7.

Animals were purchased from Harlan Olac, U.K., and group-housed in batches of four. They were kept on a 12 hour light:dark cycle (lights on at 7.30

am and off at 7.30 pm) and at an ambient temperature (19-21^{0}C) with access to standard rat feed and water *ad libitum* . All animal procedures were carried out in agreement with UK Home Office (Scientific Procedures (Animals Act) 1986) guidelines on animal experimentation and conducted by authorised personnel. The study design and dosing regime was similar to that described above in section 4.3. Thirty two young adult (2 months old) male Wistar rats (Harlan Olac, UK) were randomly assigned to either a corticosterone or vehicle treated group (mean initial body weight ± SD= 217g ± 10.7g; range 190-234g; n=16 per treatment group). Treatment began after a 7 day acclimatisation period, during which time the animals were regularly handled. Corticosterone was dissolved in ethanol as previously described (Rees et al., 2004) and added to drinking water (400 µg/mL) for 21 days according to Magarinos et al. (1998). A parallel group of rats received an equivalent amount of ethanol added to their drinking water (0.8%). Animals were as described in figure 4.1. Except that here, animals were housed in cages of four animals. Corticosterone and vehicle drinking solutions were replaced every second day with freshly prepared solutions. All water bottles were wrapped in aluminium foil to protect against any degradation of CORT as the stability of the hormone under light was not known. Water consumption was monitored as well as individual rat weight every second day. The drinking water was also replaced with fresh solutions at this time. Animals were sacrificed after a 24 hour hormone free period during the first part of the light phase, i.e. before 11am, as described in section 4.3. During the *in vivo* dosing period, animals were informally observed for general appearance and also for signs of distress in the same way as that carried out in the feasibility study. At sacrifice, tissues were collected and used for 5-HT_{1A}, oxytocin,

vasopressin 1a and CB1 receptor autoradiography. These tissues were also used for HPLC determination of 5-HT and 5-HIAA. Whole brains were either frozen in isopentane on dry ice (-40^{0}C) for autoradiography or dissected into the following 8 regions: hypothalamus, prefrontal cortex, frontal cortex, striatum, amygdala, dorsal hippocampus, ventral hippocampus, and raphé. Each region was placed into a microcentrifuge vial and snap frozen in isopentane on dry ice (-40^{0}C). Whole brains were cryosectioned into 20 µm sections as described later in section 4.4.2.3. Brain tissue sections and dissected regions were kept frozen at -80^{0}C until analysis.

Additionally, for radioimmunoassay of peptide concentration (presented in chapter 6), an additional and separate group batch of animals was included. This additional group (n=8 for vehicle treatment and n=10 for corticosterone treatment) underwent the same dosing regime as described before, but the animals were sacrificed by decapitation without anaesthesia (this was removed from the protocol in case it had an impact on the peptide concentration) and also without a 24 hour hormone free period. The rationale for sacrifice without a 24 hour hormone free period was to account for the short half-life of the neuropeptides; 1-6 minutes for oxytocin (Grewen et al., 2010) and ~10-35 minutes for vasopressin (Delmas et al., 2005), and the fact that there was no evidence suggesting that oxytocinergic and vasopressinergic effects are still evident after a 24 hour CORT washout period. Therefore, in this study, there were two timepoints considered for RIA experiments; a 0hr group that was sacrificed immediately after withdrawal of corticosterone and a 24hr group that was sacrificed after a 24 hour hormone free period. Sacrifice of animals began during the first part of the light phase and was completed by 11am (as described

in section 4.3). A statistical analysis of the water consumed, weight gained, adrenal and thymus gland weights was carried out to test for consistency between this group of animals and the previous group. This can be found in appendix B.

Whole trunk blood was collected into pre-heparinised chilled vials containing a protease inhibitor (500 KIU/mL blood of Aprotinin) to protect the peptides from degradation (Delmas et al., 2005; Grewen et al., 2010). Blood was immediately spun to obtain plasma (3 min x 13,000g) and frozen. Whole brains were dissected into the following 8 regions: pituitary gland, hypothalamus, prefrontal cortex, septal nuclei, amygdala, dorsal hippocampus, ventral hippocampus and raphé and snap frozen as previously described. Brain tissue as well as plasma samples were kept frozen at -80^{0}C until analysis.

4.4.2 Autoradiography

Autoradiography is a radiological technique that is used for the identification and localisation of a protein of interest. It is commonly used to visualise receptors in tissue samples, however it is dependent on the availability of a radiolabelled ligand. In this thesis, autoradiography is used to identify the 5-HT_{1A}, oxytocin, vasopressin 1a and CB1 receptors after chronic exposure to corticosterone. The protocols used for each experiment and analysis of data obtained are given in chapters 6, 7 and 8. Here, I describe the theory of autoradiography.

4.4.2.1 Principles of autoradiography

The principles of autoradiography are based on receptor-ligand interactions using a radiolabelled ligand for the system under investigation (Knoche, 1991). The advantage of autoradiography, over other histological techniques such as immunohistochemistry, is the fact that amount of radioligand bound can be quantified. This can be done by deducing the specific binding of a radioligand and is based on the following formulae:-

Specific binding = Total binding – non specific binding

Where: Specific binding = amount of the radioligand bound to the target of interest.
Non-specific binding = non displaceable binding to other sites (receptors, lipids or cell membranes).
Total binding = encompasses both specific and non-specific binding.

Radioligand binding data must be normalised for the amount of non-specific binding as failure to do so, may result in an overestimation of specific binding. For autoradiographical procedures, this can be achieved by the addition of saturating concentrations of unlabelled ligand to the incubation buffer. The unlabelled material competes with the radiolabelled compound, to provide a measure of background (non-specific binding) for the radioligand (Knoche, 1991).

A wide range of radionuclides (unstable isotopes which emit energy as they decay) are employed i.e. ^{3}H, ^{14}C, ^{35}S, ^{32}P, ^{11}C, ^{18}F, ^{125}I and ^{133}I. There are several steps to an autoradiography experiment and these are outlined below, summarised from Knoche, (1991):

a) Tissue Preparation:- Typically, frozen tissue is cryosectioned into thin slices and mounted onto glass polysine or gelatine coated slides. The thickness of the slices cut depends on the sensitivity and resolution required. For tritium compounds, the standard section thickness is between 10-20 µM, this is due the low penetrability of the [^{3}H] radioisotope. This thickness also provides optimal sensitivity and resolution with [^{3}H] and [^{125}I] labelled ligands and film-based visualisation techniques.

b) Incubation:- The radioligand is added to physiological buffer such as Tris(hydroxymethyl)aminomethane (TRIS). This forms the basis of an incubation buffer. In some instances, it may be necessary to add salts such as magnesium chloride or sodium chloride etc. to allow optimal conditions for binding. The sections are incubated in this solution. The incubation time varies for each target and is related to the time taken for the radioligand to obtain a state of pseudo equilibrium i.e. when the formation of the radioligand complex is equal to its dissociation.

c) Washing:- The incubation stage is terminated by rinsing the sections in ice-cold washing buffer. The purpose of this step is to remove any unbound/free radioligand.

d) Exposure to film:- The slides are dried before exposure to x-ray film which is essentially a plastic sheet that has an emulsion coating on one side. This emulsion comprises of a gelatin matrix that has either small silver halide or silver bromide crystals embedded into it. Gelatin is an

important component of the emulsion, as it ensures that each crystal is spatially segregated from adjacent crystals and that these are uniformly distributed across the emulsion. It also protects the crystals during the developing and fixing stages. Factors such as the crystal number and size are physical characteristics associated with the x-ray film, and determine what application the film may be used for (Knoche, 1991). Films such as Hyperfilm ^{3}H and Hyperfilm ßmax have a high silver content and fine grain size, making them ideal for autoradiography. As the radioisotope from the specimen underlying the film decays, it emits energy. This is absorbed by the crystals and they become activated i.e. silver ions (Ag^{+}) are converted into silver atoms (Ag^{0}). Several silver ions are converted to silver atoms in response to a single emission. For this reason, areas of the specimen which contain high amounts of radioactivity result in a greater degree of activation on the film. This results in the formation of a latent image, which cannot be seen until the film is developed.

e) Film development**:-** This is the process by which the remaining Ag+ ions within a crystal are converted to metallic silver. It is an important step as the latent image formed during exposure cannot be seen unless it is amplified in this way. The film is immersed into a developing fluid, which usually consists of a mixture of reducing agents in an alkaline solution. This results in the reduction of silver ions to metallic silver of the crystals which already contain Ag^{0} atoms and in the formation of a 'negative visual image', where the blackening of the film is directly proportional to the amount of radiation reaching it. The developing

process is time-dependent and must be stopped in time, as eventually all of the crystals present in the film will convert to metallic silver. Usually, a diluted solution of acetic acid can be used to stop the development process and also neutralise the film.

f) Film fixation:- During this step, the film is immersed into a fixing solution. The purpose of which is to remove any remaining silver bromide/halide. This step is less time-sensitive and suitable results are obtained as long as the film is in contact with the fixing solution for a long enough period of time to remove all silver ions.

Once developed and fixed, the images on the films can be analysed to provide quantitative data.

4.4.2.2 Materials used for autoradiography

All incubation reagents, developing solutions (Kodak D19 developer), fixing solutions (Kodak rapid fixer) and Radioactive film (Kodak Biomax) were purchased from Sigma Aldrich (Poole, Dorset, UK). Brains were sectioned on a Bright cryostat (Bright Instruments), sections were mounted onto polysine coated slides (Thermo Scientific). [^{3}H]microscale standards were purchased from Amersham (Buckinghamshire, UK). [^{125}I] microscale standards were purchased from American Radiolabeled Chemicals (USA).

The 5-HT_{1A} receptor radioligand used was [*0*-methyl-^{3}H]WAY 100635, specific activity: 2.81 TBq/mmol, 76Ci/mmol; radioactive concentration: 1 MBq/mL (NEN Perkin Elmer).

The oxytocin receptor radioligand used was [d(CH2)5Tyr(Me)2, Thr4,Orn8,^{125}I-Tyr-NH2(9)]vasotocin ([^{125}I]OVTA), specific activity: 81.4 TBq/mmol, 2200 Ci/mmol, radioactive concentration: 50 μCi/mL (American Radiolabeled Chemicals, USA).

The iodinated vasopressin V1a receptor radioligand was [phenylacetyl 1,0-ME-D-tyr 2 ARG 6,8,TYR 9] ^{125}I ([^{125}I]AVP), specific activity: 81.4 TBq/mmol, 2200 Ci/mmol; radioactive concentration: 0.05 MBq/mL (American Radiolabeled Chemicals, USA).

The CB1 receptor radioligand used was [^{3}H]CP 55,940 specific activity: 5.328 TBq/mmol, 144.0 Ci/mmol; radioactive concentration: 1MBq/mL (NEN Perkin Elmer).

4.4.2.3 Methodology for brain sectioning

Frozen brains collected from the *in vivo* studies described above were coronally cut into 20 μM sections in an anterior-posterior direction using a cryostat (Bright, U.K.) and collected onto polysine-coated slides. Sections were collected at the following coordinates relative to bregma according to Paxinos and Watson (5th Edn; 2005): prefrontal cortex 3.72 to 2.76 mm, dorsal hippocampus -2.92 to -3.6 mm, hypothalamus (ventral medial hypothalamic nuclei) -1.80 to -3.12 mm, paraventricular hypothalamic nuclei -1.80 to -1.92 mm, amygdala -1.92 to -3.36 mm, ventral hippocampus -5.16 to -5.88 mm, dorsal raphé and entorhinal cortex -6.96 to -8.04 mm.

4.4.2.4 Methodology for 5-HT_{1A} receptor autoradiography

On the day of analysis, slides were removed from the -80^{o}C freezer and allowed to thaw for 4 hours. Slides were placed onto two trays; one designated for determination of total binding and the other (adjacent sections) designated for determination of non-specific binding. The autoradiography protocol as described in Leventopoulos et al. (2009) was followed.

1) Sections were preincubated with Tris-HCl, pH 7.4 for 30 minutes to remove endogenous neurotransmitter and rehydrate the tissue. This was carried out by addition of 1 mL of buffer onto each slide ensuring that all tissue was covered with the solution.
2) Sections were then incubated with 2 nM of [^{3}H]WAY 100635 in Tris-HCl, pH 7.4 for 2 hours. Again, 1 mL of incubation buffer was added to each slide, ensuring that all tissue was covered.
3) Non-specific binding was determined in adjacent sections incubated in the same way but in the presence of 10 µM serotonin.
4) At the end of the incubation period, solutions were removed by pouring to waste.
5) Sections were washed by placing the slides into container filled with ice-cold Tris-HCl, pH 7.4 for 3 mins. Sections were washed in this way twice i.e. 2 x3 min washes, followed by three dips in ice-cold distilled water
6) Sections were allowed to dry overnight.

7) The following day, the slides and tritium standards were exposed to Kodak Biomax film for 6 weeks (in cassettes at-4^0C), and then manually developed using Kodak D-19 developer and rapid fixer.

4.4.2.5 Oxytocin receptor autoradiography

On the day of analysis, slides were removed from the -80^{o}C freezer and allowed to thaw for 4 hours. Slides were placed onto two trays; one designated for determination of total binding and the other (adjacent sections) designated for determination of non-specific binding. The oxytocin receptor autoradiography procedure was based on Liberzon and Young (1997) and Champagne et al. (2001).

1) Brain sections were incubated with 0.1 nM of [^{125}I]OVTA in 0.05M Tris-HCl, 0.1% BSA, 10mM $MgCl_2$, 0.05% bacitracin pH 7.4 for 1 hour at room temperature . This was carried out by addition of 1 mL of buffer onto each slide ensuring that all tissue was covered with the solution.
2) At the end of the incubation period, solutions were removed by pouring to waste.
3) Sections were washed for 8 min by placing the slides into container filled with ice-cold Tris-HCl (0.05M, pH 7.4) with 0.1% BSA, 0.01% triton-X and 100mM choline chloride. Sections were washed in this way three times i.e. 3 x8 min washes, followed by three quick dips in ice-cold distilled water.

4) Non-specific binding was determined in adjacent sections by addition of 1 mL of buffer to each slide as described but in the presence of 100 mM of oxytocin.
5) Air-dried slides and iodine-125 standards were exposed to Kodak Biomax film (in cassettes at -4^0C) for 48 hours and then manually developed using Kodak D-19 developer and rapid fixer.

4.4.2.6 Vasopressin 1a receptor autoradiography

On the day of analysis, slides were removed from the -80^{o}C freezer and allowed to thaw for 4 hours. Slides were placed onto two trays; one designated for determination of total binding and the other (adjacent sections) designated for determination of non-specific binding. The procedure was based on Campbell et al. (2009).

1) Sections were incubated with 65 pM [^{125}I]AVP in 0.05M Tris-HCl, 0.1% bovine serum albumin (BSA), 10 mM $MgCl_2$, 0.05% bacitracin pH 7.4 for 1 hour at room temperature. Incubation was conducted by addition of 1 mL of incubating buffer to the slides ensuring that all tissue sections were covered.
2) At the end of the incubation period, solutions were removed by pouring to waste.
3) Sections were rinsed for 8 minutes by placing the slides into container filled with ice-cold Tris-HCl (0.05M, pH 7.4) with 0.1% BSA, 0.01% triton-X and 100 mM choline chloride, Sections were washed in this way three times i.e. 3 x 8 min washes.

4) An additional 30 minute rinse in 0.05M Tris-HCl, pH 7.4 was carried out, followed by three quick dips in ice-cold distilled water.
5) Non-specific binding was determined in adjacent sections by incubating with1 mL of buffer in the same way as described above but in the presence of 50 μM of vasopressin .
6) Air-dried slides and iodine-125 standards were exposed to Kodak Biomax film for 48 hours (in cassettes at -4^{0}C and then manually developed using Kodak D-19 developer and rapid fixer.

4.4.2.7 CB1 receptor autoradiography

On the day of analysis, slides were removed from the -80^{o}C freezer and allowed to thaw for 4 hours. Slides were placed onto two trays; one designated for determination of total binding and the other (adjacent sections) designated for Dalton and Zavitsanou (2010) was followed.

1) Sections were incubated with 5nM [^{3}H]CP 55,940 in 0.05M Tris-HCl with 5% BSA, pH 7.4, for 2 hours at room temperature by placing 1 mL of incubation buffer onto each slide ensuring that all tissue was covered,
2) Non-specific binding was determined by incubating sections with 1 mL of buffer in the same way as described above but in the presence of 10 μM Rimonabant,
3) At the end of the incubation period, solutions were removed by pouring to waste. Sections were rinsed for 3min by placing the slides into container filled with ice-cold Tris-HCl with 5% BSA, pH 7.4,

Section were washed in this way three time i.e. 3 x 3min washes, followed by three quick dips in ice-cold water and allowed to dry overnight.

4) The following day, sections and tritiated microscales were apposed to Kodak Biomax film for 10 weeks (in cassettes at -4^{0}C) and then manually developed using Kodak D-19 developer and rapid fixer.

4.4.2.8 Film development

On the day of development, the cassettes were transported to the dark room. The developing solution was prepared by diluting the D19 developer with distilled water (1:1 v/v) and was placed into a tray. In separate trays, fixing solutions and a stop solution was placed (1L of distilled water with a drop of glacial acetic acid). Then film development took place as follows:

1) Take tape off cassette, carefully lift the film and place shiny side down into the developing tray. Agitate tray until film is covered. Leave for 1 min.
2) Remove delicately and place into stop for 1 min, making sure the film is covered.
3) Remove and place in fixer solution for 3 min
4) Once fixed, turn lights on and transfer film into bowl of distilled water.
5) Run under distilled water for at least 30 min
6) Hang up to dry in fume hood overnight

4.4.2.9 Image analysis

Images were analysed using densitometry by means of the image analysis software MCID^{TM} (version 7.0, Imaging Research Inc., Interfocus Ltd, UK). Individual regions of interest (ROIs) were identified and specific binding of the radioligand to respective receptor was measured by subtracting the non-specific binding (as determined from adjacent sections). For 5-HT_{1A} and CB1 receptor binding, where tritiated radioligands were used images were calibrated for density against commercially available microscales with decay correction to estimate radioactivity content in nCi/mg tissue. [^{3}H]radioligand binding was then converted to pmol/mg tissue. In the case of oxytocin and vasopressin 1a receptor binding, an attempt was made to calibrate images against [^{125}I]microscales, however this was not possible due to the poor quality of the commercially available standards. A non linear relationship was found to exist between concentrations of standard and relative optical density (ROD), deeming these unsuitable for quantification of images. For this reason, iodinated images were analysed using the relative optical density measure of the MCID system.

The exact locations of the ROIs sampled for 5-HT_{1A} receptor autoradiography are shown in figure 4.3 (A-E). For oxytocin receptor and vasopressin a1a receptor sampling, the ROIs sampled are shown in figure 4.4 (A-E). For CB1 receptor autoradiography, the ROIs sampled are shown in figure 4.5 (A-F). The ROI sampled has been identified by highlighting it with an orange shape on the left hemisphere. Where applicable, both left and right sides were sampled separately and data combined to give a single value. ROIs were selected as a combination of known receptor distribution, areas of dense binding

and resolution of images obtained. For example, further subregional analysis of receptor binding within these regions was possible for [^{3}H]WAY100635, [^{125}I]AVP and [^{3}H]CP 55,940 due to the resolution of autoradiography images allowing identification of subregions. However, it should be noted that subregional analysis of tissue dissected for HPLC/RIA was not possible, although the same regions were selected. For example, with autoradiography, dorsal hippocampus subregions CA1 rad, CA2 rad, CA2 pyr, CA3 rad and MODG were sampled, whereas for determination of neurotransmitter concentration, it was only possible to dissect whole dorsal hippocampus.

To enable consistency between sampling, the size of the ROIs were kept constant. Dimensions of each ROI are listed appendix C as the ROIs in figure 4.3 (A-E), figure 4.4 (A-E), figure 4.5 (A-F) are not drawn to scale.

Interassay coefficient of variation was calculated for each autoradiography experiment. A total 6 films were used for [^{3}H]WAY100635 and the interassay coefficient of variation in sampling between films was determined to be <7%. A total of 4 films each were used for [^{125}I]OVTA and [^{125}I]AVP, with an inter-assay coefficient of variation determined to be <7% for sampling between [^{125}I]OVTA films and <11% for sampling between [^{125}I]AVP films. A total 5 films were used for [^{3}H]CP 55,940 and the interassay coefficient of variation between films was determined to be <7%.

4.4.3 Determination of neurotransmitters in the brain

The present study measures concentrations of 5-HT, 5-HIAA (major metabolite of 5-HT), oxytocin and vasopressin in discrete brain regions after

chronic exposure to corticosterone. This was conducted using either high performance liquid chromatography (HPLC) or radioimmunoassay (RIA).

Here, first the theoretical background of each technique is described i.e. HPLC in section 4.4.3.1 and RIA in section 4.4.3.2 This is followed by detailed materials and methods for experiments which are presented later in chapters 5 and 6.

4.4.3.1 High performance liquid chromatography

High performance liquid chromatography (HPLC) is an established analytical technique which is popular for the separation and quantification of analytes from a range of matrices. The technique has evolved considerably and currently there are many different forms of chromatography. In general, however, the components of an HPLC system are the same. A highly polished narrow stainless steel tube which has been packed with small sized porous silica particles (3-10µm) termed the stationary phase; a buffer solution –mobile phase which carries the mixture of analytes through the system; a pump which enables the movement of the mobile phase through the system at a constant flow rate and a detector that enables identification of the analytes. Essentially, separation of a mixture occurs as a result of interactions between the analyte of interest and the stationary phase as it travels in the mobile phase.

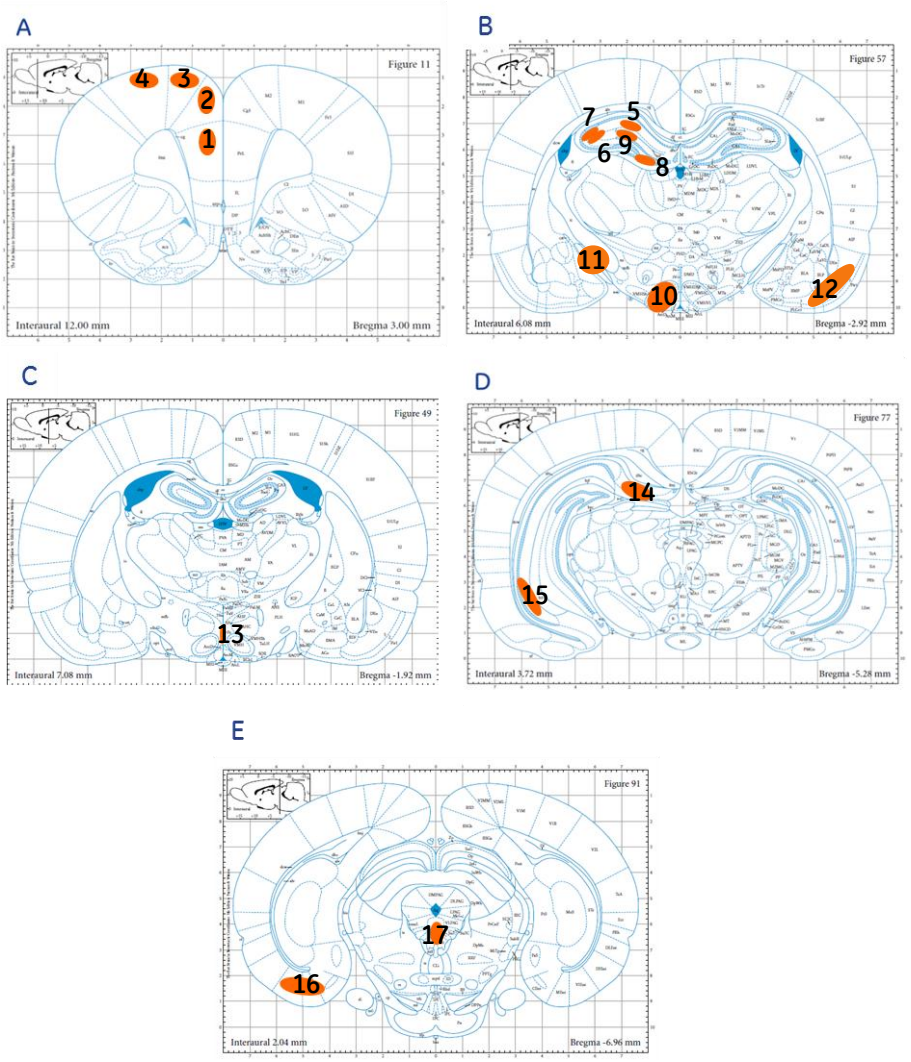


Figure 4.3 (A-E) –Location of ROI's sampled for 5-HT_{1A} receptor autoradiography. Representative images taken from the Paxinos and Watson Rat Brain Atlas 5th Edn showing regions sampled for [^{3}H]WAY 1000635 autoradiography, where 1= PRL (prelimbic cortex), 2= (CGL) cingulate cortex, 3= M2 (motor cortex), 4= M1 (motor cortex), 5= CA1-rad (radial layer of field CA1 of dorsal hippocampus), 6= CA2-rad (radial layer of field CA2 of dorsal hippocampus), 7= CA2 pyr (pyramidal layer of field CA2 of dorsal hippocampus), 8= CA3-rad (radial layer of field CA3 of dorsal hippocampus), 9= MODG (dentate gyrus of dorsal hippocampus), 10= hypothalamus, 11= amygdala, 12= pir 3 (third layer of piriform cortex), 13= PVN (paraventricular hypothalamic nuclei), 14= subiculum, 15= CA1-rad (radial layer of field CA1 of ventral hippocampus), 16= entorhinal cortex, 17= DRD (dorsal raphé nucleus).

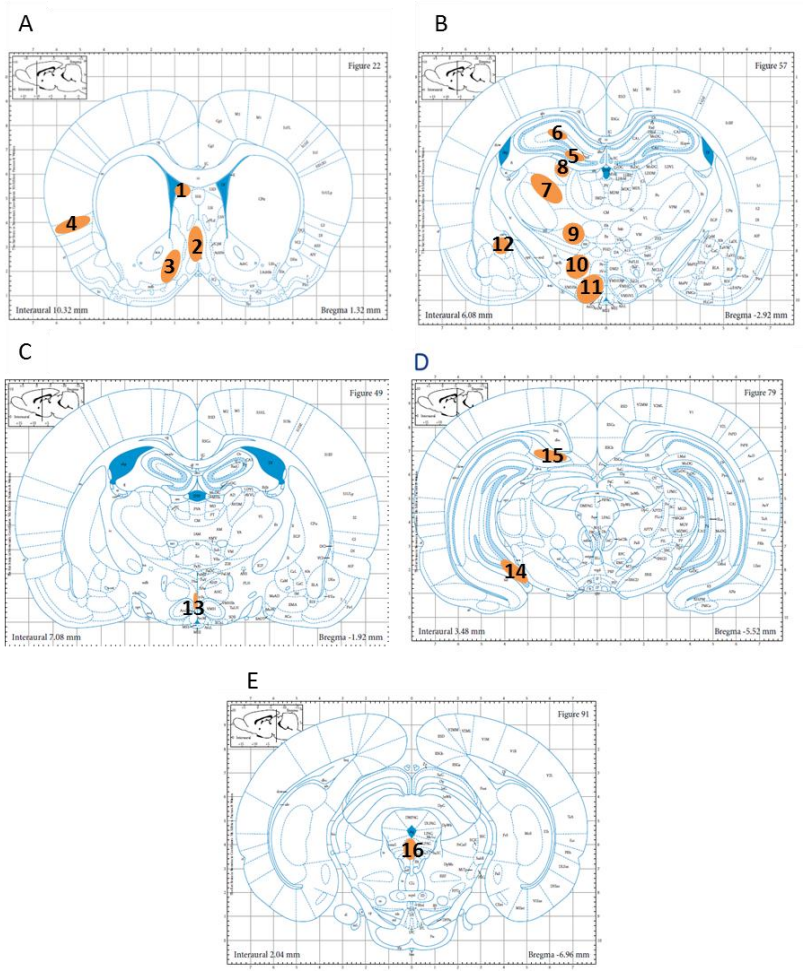


Figure 4.4 (A-E). Location of the ROIs sampled for oxytocin and vasopressin 1a receptor autoradiography. Representative images taken from the Paxinos and Watson Rat Brain Atlas 5th Edn showing the regions sampled for oxytocin and/or vasopressin 1a receptor autoradiography, where, 1= LSD (lateral septal nuclei), 2= MS (Medial septal nuclei), 3= Nucleus accumbens (shell), 4 = GI (granular insular cortex), 5 = PODG (polymorph layer of the dentate gyrus), 6= MODG (molecular layer of the dentate gyrus), 7= PO (posterior thalamic nuclear group), 8= LDDM (lateral dorsal nuclei), 9= VM (ventromedial thalamic nuclei), 10= PEFLH (perifornical lateral hypothalamus), 11= VMDH (dorsal part of ventromedial hypothalamic nucleus), 12= CeA (central amygdala nucleus), 13= PVN (paraventricular hypothalamic nuclei), 14= GrDG (Granular layer of the dentate gyrus), 15= DS (subiculum), 16= DRD (dorsal raphé nucleus).

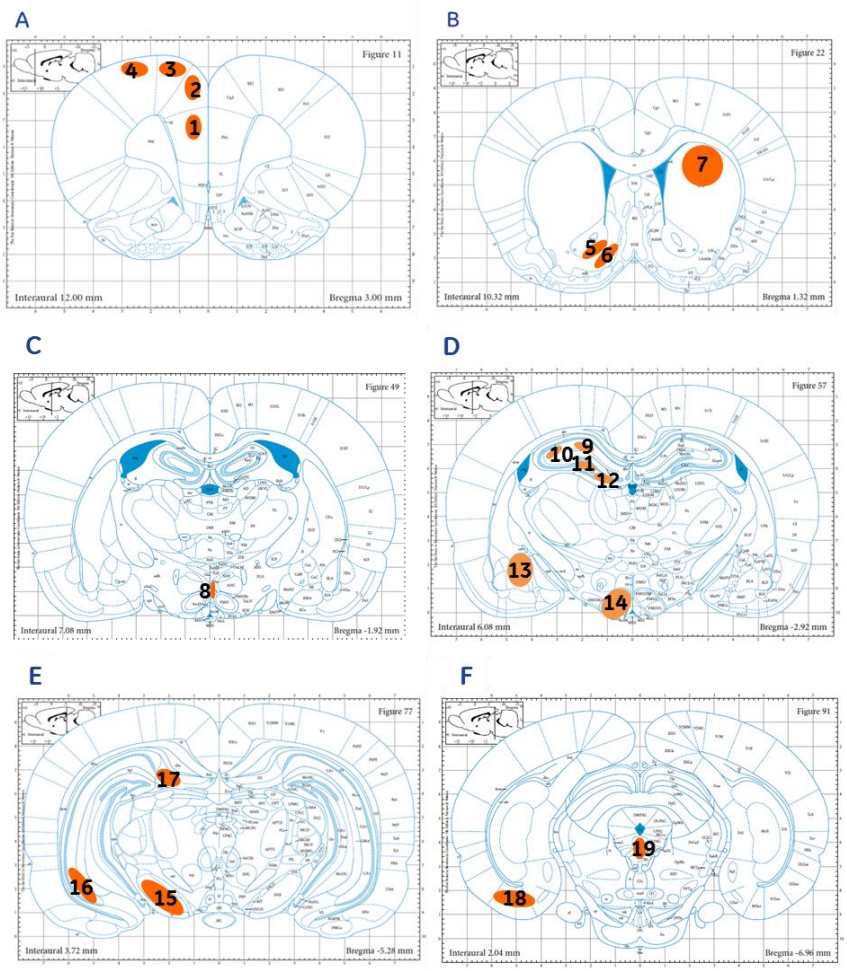


Figure 4.5 (A-F) –Location of ROIs sampled for CB1 receptor autoradiography. Representative images taken from the Paxinos and Watson Rat Brain Atlas 5th Edn showing regions sampled for [^{3}H]CP 55,940 autoradiography, where 1= PRL (prelimbic cortex), 2= (CGL) cingulate cortex, 3=M2 (motor cortex), 4= M1 (motor cortex), 5= AcbC (nucleus accumbens core), 6= AcbSh (nucleus accumbens shell), 7=striata, 8= PVN (paraventricular hypothalamic nuclei), 9= CA1-rad (radial layer of field CA1 of dorsal hippocampus), 10 = CA2-rad (radial layer of field CA2 of dorsal hippocampus), 11= CA3-rad (radial layer of field CA3 of dorsal hippocampus), 12=PODG (polymorph layer of dentate gyrus dorsal hippocampus), 13= amygdala, 14= VMDH (ventromedial hypothalamic nuclei), 15= Substantia nigra, 16=CA1-rad (radial layer of field CA1 of ventral hippocampus), 17= Subiculum, 18= entorhinal cortex , 19=DRD (dorsal raphé nucleus).

One adaptation of the original technique is reversed-phase HPLC and this has proved particularly useful for isolating neurotransmitters. Others types include absorption, ion exchange, size exclusion and gel permeation, each differing in the stationary phase used (Holman, 1993). In reversed-phase HPLC the silica stationary phase has been modified by addition of a covalently bonded hydrophobic moiety usually an n-alkyl chain C8 or C18. The mobile phase is more polar than the stationary phase and hence polar (hydrophilic) compounds are eluted faster than non-polar (hydrophobic) compounds (Holman, 1993).

The sample to be analysed is introduced to the top of the column. As the sample moves through the column, the individual components are differentially retained on the stationary phase (a process that is dependent on the strength of interaction between the analyte and the hydrophobic moieties of the stationary phase). In this way, components of the sample are eluted from the column at different times. These are referred to as the retention times and are defined by the time elapsed between injection onto the column and being detected. There are many factors that will influence the retention time of a compound such as type of stationary phase, mobile phase composition, pH and temperature which may affect the separation of analytes (Isimer et al., 1991). In addition, organic modifiers and anion pairing agents can be added to the mobile phase that alter the retention times of compounds allowing more control over the separation. For example, in the context of indole analysis, the separation of 5-HT from its more acidic metabolite, 5-HIAA can take a long time. However, through addition of methanol (organic modifier of the mobile phase), it is possible to speed up the overall runtime. Furthermore, octane sulphonic acid (anion pairing agent) strengthens the interaction of amines with the stationary phase and so increases

their retention time (Isimer et al., 1991). HPLC is a highly sensitive technique and the parameters mentioned above must be kept constant as even small changes in any of these can be detrimental to the analyses (Isimer et al., 1991).

As the analytes are eluted from the column, they pass through a detector which has been programmed to identify the analyte of interest based on one of several properties, for example ultraviolet (UV), fluorescent or electrical signals (Stein, 1982). Electrical chemical detection (ECD) is one of the most sensitive detection methods for HPLC and is often used in studies measuring neurotransmitter concentrations. ECD relies on the generation of a potential difference between the working electrode and the mobile phase. The detectors can be set in an oxidation or reduction mode. In the oxidation mode, any component of the mobile phase (including analyte of interest) which can be oxidised will pass electrons (and hence current) from the mobile phase to the working electrode. The flow of current is directly proportional to the concentration of the analyte and so quantification of each analyte can be attained (Stein, 1982).

4.4.3.2 Materials used for HPLC experiment

All mobile phase reagents, extraction solvents (perchloric acid and EDTA) and indoleamine standards were purchased from Sigma Aldrich (Poole, Dorset, UK). The HPLC hardware system used was the ESA, model 582 solvent delivery system, with an ESA Coulochem II electrochemical detector coupled to the EZChrom Data Capture and Analysis software. Extracts were filtered through a syringe filter (Millipore) and placed into light protective chromacol vials and inserts (Chromacol) before separation of components on an analytical

HPLC column (5µM Spherisorb ODS, 4.6 x 100mm analytical column (Waters Analytical)).

4.4.3.3 Methodology for Indole determination by HPLC

Prior to tissue analysis, the HPLC method was optimised. Standards were generated from 5mM stock solutions, ranging between (0.03-5.00 µmol/L). Rat brain tissue collected during the feasibility study was used here for method development and optimisation. Indoleamines extracted from striatal and frontal cortex tissue were analysed at 2 µA with a standard curve in the range of 0.1-5 µmol/L, whereas a higher sensitivity (0.5 µA; standard curve range 0.025-1.25 µmol/L) was required for hypothalamus, prefrontal cortex, amygdala, dorsal hippocampus, ventral hippocampus and raphé, due to a combination of lower indoleamine content and smaller tissue mass of these regions. A typical standard curve from the validation of striatal and prefrontal cortex tissue analysis is shown in figure 4.6. The higher sensitivity range standard curve used for the remaining tissues is shown in fig 4.7.

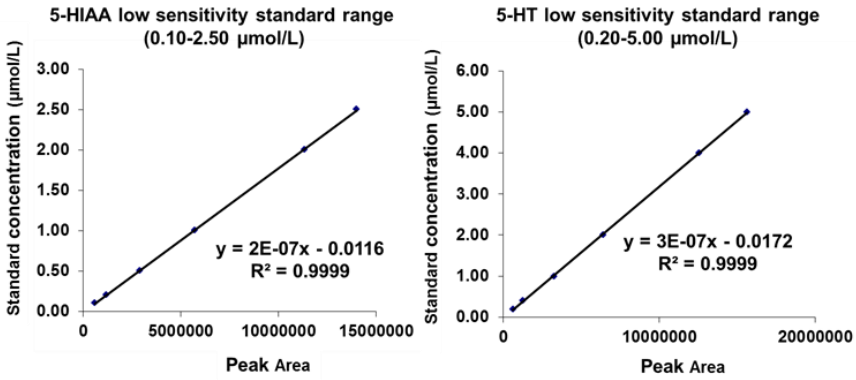


Figure 4,6 Calibration curves from the lower sensitivity range of standards for 5-HIAA and 5-HT (0.10-5.00 µmol/L). This range of standards was used to quantify 5-HIAA and 5-HT from frontal cortex and striata tissue from both vehicle and CORT treated rats.

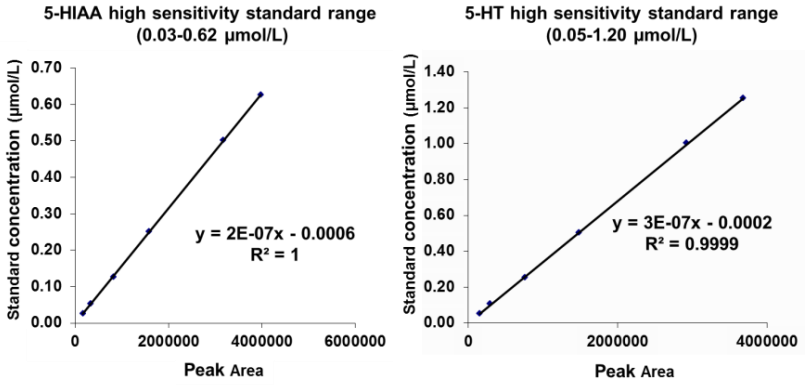


Figure 4.7 Calibration curves from the higher sensitivity range of standards for 5-HIAA and 5-HT (0.03-0.62 µmol/L). This range of standards was used to quantify 5-HIAA and 5-HT content in hypothalamus, prefrontal cortex, amygdala, dorsal hippocampus, ventral hippocampus and raphé tissue obtained from both vehicle and CORT treated rats.

Once the methodology was optimised, the following protocol was followed for extraction and sample preparation:

1) Frozen tissue from the current study was weighed and immediately homogenised (sonicated for 20 seconds) in ice-cold 0.1M perchloric acid/0.5mM EDTA (Zaczek and Coyle 1982).
2) Tissues were kept on ice for 20 minutes before centrifugation (3,000 rpm, 4^{o}C for 20min).
3) The resulting supernatant was filtered through 0.4 µm filters (Millipore, U.K.) and 200 µL of the supernatant was placed into chromacol vials.
4) A 50 µL aliquot was analysed on a 5 µm Spherisorb 4.6 x 100 mm column (Waters Ltd, UK.).

The mobile phase consisted of 0.05M NaH_2PO_4, 1 mM OSA, 0.1 mM EDTA, 10% MeOH, pH 3.2 at a flow rate of 1mL/min. The electrochemical detector settings were as follows: screening electrode 0mV, analytical electrode +300 mV and guard cell -600 mV. The sensitivities of the analytical electrode ranged from 0.5 µA to 2 µA, depending on the brain region analysed. Tissue concentrations of 5-HT and its metabolites 5-HIAA were quantified by external standard curve calibration as explained above, using peak areas for quantification. Fresh standard solutions were prepared daily. Data were converted and expressed as pmol/mg tissue.

4.4.4 Radioimmunoassay

Radioimmunoassay (RIA) is a technique which is used to measure concentrations of hormones in a sample. It is a highly sensitive and specific

technique allowing detection of analytes in the picogram range (Skelley et al., 1973). It is based on the principle of ligand-antibody binding, where the antigen (e.g. hormone of interest) binds to the antibody to form a complex as described by the equation below:

$$Ag + Ab \underset{k_i}{\overset{k}{\rightleftharpoons}} AgAb \qquad (1)$$

Where: Ag = antigen
Ab = antibody
AgAb = antigen-antibody complex
k= rate constant for association
ki=rate constant for dissociation

At equilibrium, the concentration of AgAb is the same as the concentration of Ag and Ab individually,

$$K[Ag][Ab] = [AgAb] \qquad (2)$$

Where: K = equilibrium constant (k/ki)

At equilibrium, the ratio of bound fraction and free fraction can be described as:

$$B/F = [AgAb]/[Ag] \qquad (3)$$

Where: B= bound fraction
F= free fraction

In order to measure B or F, a small (and constant) amount of radiolabelled antigen is introduced to set up a competition between the labelled and unlabelled antigen for the limited antibody. Then B is measured by precipitating the radiolabelled AgAb complex from the solution by the addition of a secondary antibody. After a centrifugation stage, the B fraction is represented by the pellet, whereas F remains in the supernatant. Both fractions

are counted in a gamma counter and the concentration of antigen [Ag] of interest in the sample determined. A standard curve is generated by running 'known' concentrations in parallel to 'unknowns' from which unlabelled [Ag] can be extrapolated.

4.4.4.1 Materials used for radioimmunoassay experiment

Commercially available kits were purchased for the analysis of neuropeptide concentration in plasma and tissue extracts. Separate kits were purchased for oxytocin and vasopressin (Phoenix Pharmaceuticals Inc.; product code RK-051-01, RK-065-07 respectively). The oxytocin kit had a detection limit of 30 pg/mL (range 10-1280 pg/mL). The inter-assay and intra-assay variation was not measured in this study, however, based on published literature the oxytocin kit had an inter-assay and intra-assay variation of 9% and 11% respectively (Marrazziti et al., 2006). The vasopressin kit had a detection limit of 31.1 pg/mL (range 10-1280 pg/mL). Again, the inter-assay and intra-assay variation was not measured in this study, however, based on published literature the vasopressin kit had an inter-assay and intra-assay variation of <9% and <12% respectively (Oritz et al., 2002). The cross reactivity of the oxytocin kit was 100% with oxytocin and 0% with Lys-vasopressin, Arg-vasopressin, growth hormone, alpha-ANP, Met-Enkephalin, gonadotropin releasing factor, somatostatin, thryotropin releasing hormone, vasoactive intestinal peptide, Pacap 27-NH2. Whereas, the cross reactivity of the vasopressin kit was 100% with [Arg^8]-Vasopressin, [Arg^8]-Vasotocin and vasopressin metabolite neuropeptide, 38% with [Lys^8]-Vasopressin, 0.8% with Deamino-[D-Arg^8]-

Vasopressin and 0% with α-ANP (1-28), Oxytocin, ACTH, LH-RH, Met-Enkephalin, PACAP-38, PACAP-27 Amide and Pressionic Acid.

Plasma samples were measured after solid phase extraction using Sep pak cartridges Strata-X, C18; 3 x 300 mg/mL (Phenomenex).

A commercially available kit was also used for the determination of corticosterone in plasma (MP Biomedicals; product code 07120102). Plasma corticosterone concentration was measured directly without prior extraction as instructed by the kit protocol. The minimum detectable amount of corticosterone using this kit was 7.7 ng/mL. The intra-assay variation and inter-assay variation was quoted to be <10% and <7% respectively (MP Biomedicals). The cross reactivity of the kit was 100% with corticosterone, 0.34% with deoxycorticosterone, 0.1% with testosterone, 0.05% with cortisol, 0.03% with aldosterone, 0.02% with progesterone, 0.01% with androstenedione, 0.01% with 5-α dihydrotestosterone, <0.01% with cholesterol, dihydrotestosterone, dehydroepiandrosterone sulphate, 11-desoxycortisol, dexamethasone, 20α-dihydroprogesterone, estrone, estradiol-17α, estradiol -17β, estriol, pregnenolone, 17α-hydroxypregnenolone and 17α-hydroxyprogesterone.

4.4.4.2 Methodology for determination of peptide concentration by radioimmunoassay

Oxytocin and vasopressin concentrations were measured in dissected brain regions using radioimmunoassay with prior extraction of peptides as detailed in Stabile et al., (2010). This is outlined in more details below:

1) On the day of extraction, samples were removed from the -80^{o}C freezer but kept packed on dry ice.
2) Each brain region was individually weighed before addition of 0.1M acetic acid (200 µL for each region except prefrontal cortex, where 800 µL was added due to the greater tissue mass).
3) The sample vials were then immediately placed into a bath of boiling water for 20 minutes.
4) After 20 mins, sample vials were removed and allowed to cool to room temperature.
5) The vials were then frozen at -20^{o}C (tissue debris and acetic acid extract together) until analysis.

Plasma quantification of oxytocin and vasopressin was carried out after solid phase extraction (SPE) of the peptides from acidified plasma samples using Strata X, SPE cartridges (C18; 3 x 300 mg/mL; Phoenix Pharmaceuticals Inc.). This was achieved using the reagents and protocol supplied in the RIA kit and is outlined below:

1) Dilute the plasma samples by addition of 500 µL of buffer A to 500 µL of plasma.
2) The SPE cartridges were equilibrated by addition of 1 mL buffer B and then 3 x 3 mL of buffer A.
3) The acidified plasma sample (1 mL) was then loaded onto the column.
4) The column was then washed with 2 x 3 mL of buffer A.
5) The peptides were then eluted by addition of 3 mL of buffer B.
6) The eluent was collected and evaporated to dryness in a centrifugal concentrator and resuspended in 250 µL of RIA buffer.

Tissue extracts were analysed as outlined in the RIA kit and is described below:

1) 100 µL of extract was added to 100 µL of primary antibody (rabbit anti peptide serum),
2) The samples were vortexed and incubated at 4^0C for 24 hours.
3) Then 100 µL of [^{125}I] peptide was added and samples were incubated for 24 hours at 4^0C.
4) The following day, 100 µL of goat anti rabbit IgG serum and normal rabbit serum was added to each tube.
5) Samples were vortexed and incubated for a further 90 minutes at room temperature
6) 500 µL of RIA buffer was added to each tube and centrifuged (3,000 rpm for 20 minutes at 4^0C) to separate bound and unbound radiolabelled fractions.
7) The supernatant was immediately aspirated and both supernatant and pellet were counted in a gamma counter.

4.4.4.3 Corticosterone Radioimmunoassay

The concentration of corticosterone in plasma samples was measured using a commercially available kit from MP Biomedical. The samples were analysed directly without extraction as instructed by the kit protocol to give a corticosterone concentration in ng/mL. Plasma samples were measured in duplicate, the intraassay variation in this study was <11%. This is outlined below:

1) Plasma samples were diluted 1:200 with steroid diluent,

2) [^{125}I]corticosterone was added before introduction of antiserum (anti corticosterone).
3) The samples were vortexed and incubated at room temperature for 2 hours, before addition of the precipitant.
4) The samples were vortexed again and centrifuged at 2,300 rpm for 15 minutes.
5) Both pellet and supernatant fractions were counted in a gamma counter.

4.5 Statistical analysis

Data were analysed using GraphPad Prizm 4.0 (Sigma Aldrich, Dorset UK). All data presented in this thesis passed the Kolmogorov-Smirnov test for normality.

For effects of corticosterone treatment on consumption of drinking water and body weight, data were analysed by means of two-way ANOVA for effects of treatment and duration of treatment. If the ANOVA result showed significance, individual data from each time point was analysed separately using Student's two-tailed t-test (unpaired) with Bonferroni post hoc test applied to correct for multiple comparisons. The significance level was set at $P = 0.05$. Autoradiography, HPLC and RIA (peptide content) data was also analysed using a two-way ANOVA for effects of treatment and region. Plasma corticosterone data were also analysed using a two-way ANOVA for effects of treatment and timepoint of sacrifice (i.e. immediately after withdrawal of CORT or after a 24 hour period).

Where the ANOVA showed significant effects, data were analysed separately with Student's two-tailed t-test (unpaired) with Bonferroni post hoc

test applied to correct for multiple comparisons. The significance level was set at P = 0.05. Post mortem adrenal and thymus gland weights were analysed by Student's two-tailed t-test (unpaired) with a significance level set at P = 0.05.

All data presented in this thesis are expressed as mean ± SD in the text and tables, and are shown as mean ± SEM in graphs. Statistical differences assessed as described above are denoted by asterisks *.A record of the statistical analyses can be found in appendix B.

Chapter 5

Serotonergic responses to chronic glucocorticoid exposure

Glucocorticoids serve to maintain homeostatic control via a negative feedback mechanism, however, when this is dysfunctional an allostatic load occurs. Approximately 50% of depressed patients also show hyperactivity of the HPA axis in addition to serotonergic dysfunction (Holsboer, 2000; Pariante and Miller, 2001; Pariante, 2003; Anacker et al., 2011). In this chapter, I investigate the effect that chronically elevated glucocorticoids have on central 5-HT_{1A} receptor binding and serotonin turnover and discuss how these changes may be related to glucocorticoid induced dysfunction associated with stress related disorders.

5.1 Introduction

It is well known that stressful life experiences will contribute to the aetiology of depression and anxiety related disorders, and this relationship has been studied extensively (Kendler et al., 2008; reviewed in Lanfumey et al., 2008). Overactivity of the HPA axis arising from the loss of negative feedback is an important hallmark of these disorders (Anacker et al., 2011). In addition, aberrant serotonergic signalling has also been described in depressed patients with an underlying HPA axis dysfunction (Mikkelsen, 2004; Anacker et al., 2011). Thus, the involvement of serotonin in the regulation of the stress response has been the subject of intense interest (Baganz et al., 2010; Goel et al., 2011; Curran and Chalsani, 2012). In particular, it is thought that an individual's response to antidepressants may be dependent on differences in their HPA axis responsiveness and also to the degree of abnormalities in the serotonergic system (Anacker et al., 2011). This would provide one explanation for the high proportion of depressed patients which are treatment-resistant, although it is likely to be a combination of many other factors too such as involvement of the GR and other neurotransmitter systems (Anacker et al., 2011).

At a molecular level, interactions between the serotonergic and glucocorticoid systems have been elucidated (Falkenberg and Rajeevan, 2010). Serotonergic influence on the HPA axis is implicated at the level of the hippocampus, hypothalamus, pituitary and adrenal glands (Chaouloff, 1993; Falkenberg and Rajeevan, 2010). Whilst serotonin activates the HPA axis resulting in glucocorticoid secretion, the activation of the 5-HT_{1A} receptor can be considered to be anxiolytic under normal conditions, playing a role in the negative feedback mechanism alongside the glucocorticoid receptor (Chaouloff,

1993). However, in depression, serotonergic signalling at the 5-HT_{1A} receptor is impaired, and in conjunction with GR dysfunction, this contributes to HPA axis hyperactivity by inhibiting the glucocorticoid-mediated negative feedback mechanism (Mikkelsen et al., 2004; Anacker et al., 2011). In addition, in the hypothalamus the 5-HT_{1A} receptors are expressed by CRH neurones. The release of CRH from the hypothalamus is independent of hypothalamic GR receptors; therefore a role for the 5-HT_{1A} receptors in regulating the HPA axis is postulated (Falkenberg and Rajeevan, 2010).

Experimentally, exogenous corticosterone can be administered to achieve long-term increases in peripheral concentrations in order to study their neurobiological effects. In this way, chronic administration of corticosterone has been shown to induce anxiety and depression-like behaviour in rodents (Ardayfio and Kim, 2006). Chronic exposure to an elevated concentration of glucocorticoid results in changes in central serotonergic regulation, namely a reduction in 5-HT_{1A} receptor binding in the hippocampus, cortex and raphé and increased serotonin release (Meijer et al., 1997; Lopez et al., 1998; Bush et al., 2003).

In this study, the response to chronic corticosterone was investigated at both the presynaptic and postsynaptic 5-HT_{1A} receptor and related to serotonin turnover in the same regions. The presynaptic receptor is predominately located on 5-HT neurone cell bodies and dendrites in the raphé region (Palacios et al., 1990) and due to the extensive innervation of the serotonergic system throughout the brain, activation of these presynaptic receptors can influence serotonergic activity in projection areas i.e. cortex, hippocampus, amygdala, which are all regions where the 5-HT_{1A} receptor is expressed postsynaptically (Palacios et al.,

1990). To our knowledge, this is the first attempt to simultaneously study the effects of chronic exposure to glucocorticoids at both the pre-and postsynaptic 5-HT_{1A} receptor and investigating receptor activity to serotonin turnover in the same region. In addition, this is the first time that the 5-HT_{1A} receptor in limbic and extralimbic regions has been investigated in the same study.

5.1.1 5-HT_{1A} receptor and the stress response

The measurement of changes in 5-HT_{1A} receptor density after chronic stress or exogenous GC administration is well documented. Chronic treatment with corticosterone reduced 5-HT_{1A} receptor binding in the cortex and hippocampus of rats (Meijer et al., 1997; Bush et al., 2003). Also, chronic immobilisation stress decreased 5-HT_{1A} receptor binding in the hippocampus as a result of increased circulating corticosterone concentration (Mendelson and McEwen, 1991; 1992). In addition, repeated stress in rats and tree shrews led to an 11%-34% reduction in 5-HT_{1A} receptor density in parietal cortex, prefrontal cortex, posterior cingulate and hippocampus (Flugge, 1995). As well as influencing the number of 5-HT_{1A} receptors, glucocorticoids affect the functionality of these receptors. Prolonged treatment of rats with corticosterone (10mg/kg s.c. given twice daily for 7 days) induced attenuated population spikes and hyperpolarisation in the hippocampus in response to the selective 5-HT_{1A} receptor agonist, 8-OH-DPAT (Czyrak et al., 2002). These corticosterone-induced changes were reversed after treatment with imipramine, a tricyclic antidepressant, which acts to increase central serotonin levels (Zahorodna et al., 2006). In addition, desensitisation of raphé autoreceptors is known to occur in response to glucocorticoids (Fairchild et al., 2003).

Interestingly, *post mortem* studies in depressed patients with hypersecretion of cortisol indicated dysfunction in the expression of the 5-HT_{1A} receptor. A reduction in hippocampal 5-HT_{1A} receptor mRNA was observed, which was attributed to cortisol-dependent inhibition of 5-HT_{1A} receptor mRNA expression (Lopez et al., 1998).

5.1.2 Serotonergic activity during the stress response

Changes in serotonergic activity can indicate abnormalities in the regulation of the HPA axis. These can be measured as changes in the synthesis and release of 5-HT, its major metabolite 5-HIAA, or serotonin turnover, defined as the ratio of 5- 5-HIAA:5-HT (Fuller and Wong, 1990). Stress-induced increases in 5-HT release in the raphé, prefrontal cortex, hippocampus and amygdala have all been previously demonstrated (Maswood et al., 1998; Amat et al., 2005). However, regional variations in the serotonergic response to stress exist. Thus, a period of forced swim increased 5-HT in the striatum of rats whereas; 5-HT concentration in the amygdala and septal nuclei was decreased or remained unchanged in the hippocampus and frontal cortex (Kirby et al., 1995). In the same way, regional specificity exists for 5-HIAA concentration measured in response to stress. Immobilisation and exposure to cold stress has been shown to cause an increase in 5-HIAA release in the frontal cortex and raphé of rats, but forced exercise stress caused an increase in 5-HIAA levels in the frontal cortex only (Clement et al., 1993). In another study, forced swimming reduced 5-HIAA concentration in all regions sampled (Kirby et al., 1995).

With this in mind, is not surprising that regional variations in the effect of stress on serotonin turnover are also seen. Tail pinch induced stress caused an

increase in serotonin turnover in the frontal cortex and hippocampus of rats, but not in the striata, olfactory tubercles or hypothalamus (Pei et al., 1990). Interestingly, the intensity of a stressor can be an important determinant of the serotonergic response. A high intensity electric foot shock produced increases in 5-HT turnover in the prefrontal cortex, nucleus accumbens and hypothalamus, whereas no changes were measured after a low intensity electric shock in the same regions (Inoue et al., 1994). Furthermore, it has been demonstrated that the increased serotonergic neuronal activity in the raphé that results after exposure to stress can be attributed to an increase in 5-HT turnover and release rather than to an increase in neuronal firing rate (Takase et al., 2004), which is an effect that may arise from the stimulation of tryptophan hydroxylase by corticosterone (Lanfumey et al., 2008).

In this chapter, I describe experiments that were designed to evaluate changes in 5-HT_{1A} receptor binding after chronic exposure to exogenous corticosterone in male Wistar rats. In addition, I quantified the concentration of 5-HT, 5-HIAA and calculated serotonin turnover in discrete brain regions of CORT treated rats. Autoradiography was used to determine binding of [^{3}H]WAY 100635 to 5-HT_{1A} receptors in rat brain. The highly selective antagonist radioligand (Ki = 0.85 nM) has been extensively used to study the 5-HT_{1A} receptor (Forster et al., 1995; Khawaja et al., 1995). Changes in serotonin turnover were measured by quantifying 5-HT and 5-HIAA in tissue homogenates representing total (intracellular and extracellular) tissue concentrations of each, and calculating the ratio of 5-HIAA to 5-HT (Hirani, 2006).

5.2 Aims and Hypothesis

5.2.1 Aims

The main objective of the programme of work is to establish the effect of chronic exposure to corticosterone, as a model of chronic stress, on the serotonergic system in the brain. It has been reported that high corticosterone levels reduce 5-HT mediated inhibition and excitatory response to phenylephrine, an α-adrenergic receptor agonist (Judge et al., 2004). Czyrak et al., 2002 observed that prolonged treatment with corticosterone (10mg/kg s.c. given twice daily for 7 days) induced attenuated population spikes and hyperpolarisation in hippocampus in response to 8-OH-DPAT, a selective agonist at the 5-HT_{1A} receptor. These corticosterone-induced changes were reversed after treatment with imipramine, a tricyclic antidepressant, which acts to increase central serotonin levels (Zahorodna et al., 2006).

With regards to the 5-HT_{1A} receptor, reduced binding was observed in the cortex and hippocampus after chronic treatment of rats with corticosterone (Meijer et al., 1997 and Bush et al., 2003). Further support of the fact that glucocorticoids influence 5-HT_{1A} binding is a study by Chalmers et al., 1993 which showed that adrenalectomised rats show an increase in 5-HT_{1A} binding in hippocampus relative to sham operated rats. It has also been reported that elevated circulating corticosterone levels cause 5-HT_{1A} receptors to become desensitized and receptor expression decreases. It has not been studied how the glucocorticoid-dependent receptor changes correlate with serotonin neurotransmitter activity, nor has the effect of glucocorticoids on the serotonergic system been assessed in extralimbic as well as limbic regions. In order to address this gap in knowledge, serotonin turnover and 5-HT_{1A} receptor

binding will be studied in discrete brain areas post mortem.

This thesis tests and reports, for the first time, receptor and neurotransmitter level changes simultaneously from the same study. Such a study, using a dual approach has not previously been done and will determine whether the receptors altered by elevated corticosterone levels are coupled with pre-synaptic or post-synaptic regulatory effects.

5.2.2 Hypothesis

It is hypothesised that chronic treatment with corticosterone, will lead to 5-HT_{1A} receptor density changes similar to those already published in the literature. More specifically, there will be a reduction in binding at the 5-HT_{1A} receptor in the limbic brain regions implicated in responses to stress. Also, it is hypothesised that these receptor changes will be linked to serotonin turnover effects in limbic brain regions implicated in responses to stress.

5.3 Materials and Methods

All materials used for this study are given in chapter 4. Also, in chapter 4, are methodological details on in-vivo husbandry, dosing on animals, collection of post mortem samples and tissue sectioning, HPLC and autoradiography.

It should be noted that in this study, animals were sacrificed with the inclusion of a 24 hour hormone free period (van Gemert et al., 2006).

5.4 Results

5.4.1 Water consumption

Water consumption per cage was measured every second day. This was converted to volume of water consumed per rat (mL/rat) and is shown in figure 5.1 (A).

A two way analysis of variance was used to test for effects of CORT treatment on the volume of water consumed and also any effects associated with treatment duration on the volume of water consumed. Significant differences were found for duration ($F(9,60) = 5.08$, $P < 0.0001$) and treatment ($F(1,60) = 12.02$, $P = 0.001$). Further analysis between treatment groups at individual time points using two-tailed Student's t-test (unpaired) with Bonferroni correction with a significance level set at $P = 0.05$ revealed that CORT treated animals consumed less water than vehicle treated animals at day 17 of treatment. When normalised to body weight (figure 6.5 (B)), significant differences in volume of water consumed were found to be affected by duration ($F(9,300) = 17.51$, $P < 0.0001$) and treatment ($F(1,300) = 113.4$, $P<0.0001$). The interaction was also significant ($F(9,300) = 5.24$, $P <0.0001$). Further analysis between treatment groups at individual time points using two-tailed Student's t-test (unpaired) with Bonferroni correction with a significance level set at $P = 0.05$ revealed that CORT treated animals drank a greater volume of water at day 7 and then from day 13 until day 21 (end of the treatment period). The amount of CORT ingested by the treated group was calculated. Using the average volume of water consumed by the CORT treated animals (mL/rat) at the beginning and end of the treatment (day 3, 64 mL; day 21, 70.5 mL) and the weight of the animals at the

beginning and end of treatment (day 3, 213.8g; day 21, 223.0g), the range of CORT ingested was 59.9 mg/kg/day to 63.2 mg/kg/day. The average CORT dose was calculated to be 55.7 mg/kg/day (based on an average of 60.7 mL of water consumed and an average body weight of 218.0g).

5.4.2 Body weight

Animals were weighed every second day throughout the treatment period and changes in body weight are shown in figure 5.2. A two way analysis of variance was used to test for effects of treatment on weight gain and also for effects of treatment duration. Significant differences were found for both duration ($F(10,330) = 32.71$, $P = <0.0001$) and treatment ($F(1,330) = 885.4$, $P = <0.0001$). The interaction was also significant ($F(10,330) = 18.77$, $P = <0.0001$). Further analysis between treatment groups at individual time points using two-tailed Student's t-test (unpaired) with Bonferroni correction with a significance level set at $P = 0.05$ revealed that CORT treated animals showed attenuated weight gain throughout the treatment period. Vehicle treated rats steadily gained weight with an increase from 220g ± 4g to 308g ± 24g, representing a 40% increase in body weight over the duration of the study. However, CORT-treated animal's mean weight gain increased from 214g ± 14g to 223g ± 14g representing only a 4% increase in body weight over the same period. Thus, treatment with CORT for 21 days significantly attenuated weight gain in male Wistar rats. In addition, the effect of CORT on body weight was immediate, having significantly reduced the rate of weight gain by the second day of treatment.

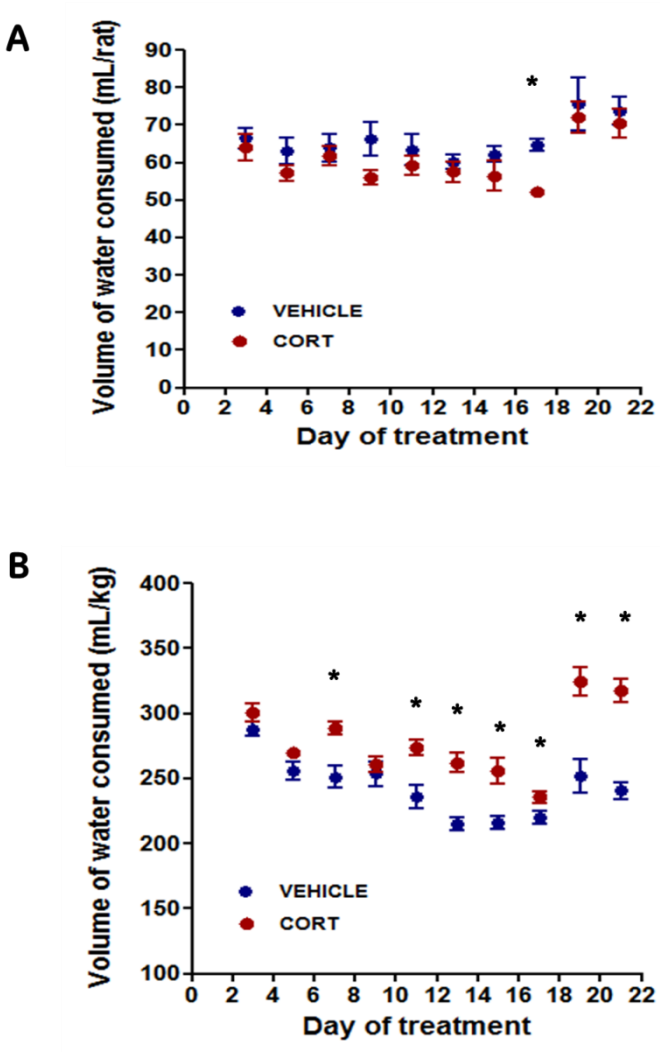


Figure 5.1. Water consumption of vehicle and CORT treated animals. Animals were treated with either 400µg/mL CORT or vehicle for 21 days. Based on the assumption that each rat consumed an equal volume of water, mean volume of water consumed per rat was calculated (A). This was then normalised to individual rat weight to give water consumption as mL /kg (B). Data are expressed as mean ± SEM of n=16 per treatment group. Statistical analysis was carried out using 2-way ANOVA for treatment and duration of treatment. A Bonferroni post hoc test for multiple comparisons was applied with at a significance level of 0.05. A statistically significant effect after Bonferroni correction is denoted by *.

5.4.3 Ex vivo readouts - adrenal and thymus gland weights

At sacrifice, adrenal and thymus glands were dissected and weighed. Absolute adrenal gland weights (left and right side combined) and thymus gland weights are shown in table 5.1 alongside tissue weights expressed as mg/100g body weight.

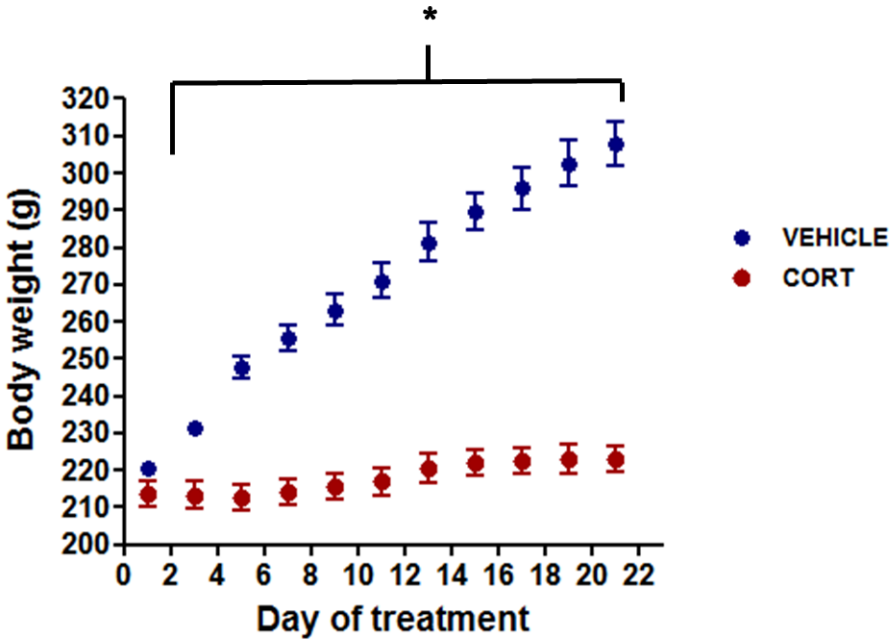


Figure 5.2. Body weight measurements of both vehicle and CORT treated animals. Animals were treated as described in figure 5.1. Each animal was weighed every second day. Data are expressed as mean ± SEM of n=16 per treatment group. Statistical analysis was carried out using 2-way ANOVA for effects of treatment duration and treatment itself. A Bonferroni post hoc test for multiple comparisons was applied with at a significance level of 0.05. A statistically significant effect after Bonferroni correction is denoted by *.

Student's two-tailed t-test (unpaired) was used to test for effects of CORT treatment on organ weights. Absolute organ weights were significantly

reduced in CORT treated animals (P<0.0001). When normalised to body weight, both glands were significantly reduced in weight after CORT treatment when compared to vehicle treated animals (P=0.0017 for adrenal glands and P<0.0001 for thymus; CORT versus control). Chronic exposure to CORT for 21 days resulted in a decrease in adrenal gland weight from 22.22 ± 3.30 mg/100g body weight in the vehicle treated group to 15.98 ± 6.54 mg/100g body weight in CORT-treated animals. This represented a 28.1% reduction in adrenal gland weight after chronic CORT treatment. Thymus weights were reduced from 162.60 ± 29.65 mg/100g body weight in the vehicle treated group to 110.80 ± 34.35 mg/100g body weight in CORT-treated animals. This represented a 31.9% reduction in thymus gland weight after chronic CORT treatment.

Table 5.1. Effect of chronic exposure to CORT on thymus and adrenal gland weights.

Organ	Vehicle group (organ weight (mg) /body weight (g))	CORT group (organ weight (mg) /body weight (g))	Vehicle group (absolute organ weight (mg))	CORT group (absolute organ weight (mg))
Adrenal glands	22.22 ± 3.30	15.98 ± 6.54 **	67.16 ± 10.47	34.69 ± 13.27 ***
Thymus glands	162.60 ± 29.65	110.80 ± 34.35 ***	493.20 ± 102.41	242.12 ± 69.28 ***

Table 5.1. Animals were treated in the same way as described in figure 5.1. At sacrifice, adrenal glands and thymus was dissected and weighed. Tissue weights (mg) were normalised to body weight of the animal (g) (n=16 per treatment group). Data are expressed as mean ± SD. Statistical analysis was via Student's two tailed t-test (unpaired) where **P<0.01, ***P<0.001.

5.4.4 5-HT_{1A} autoradiography

Changes in binding to the 5-HT_{1A} receptor were quantified after chronic exposure to CORT. The quantitative data showing [^{3}H]WAY 100635 specific binding to 5-HT_{1A} receptors expressed as pmol/mg tissue are shown in table 5.2. A two way analysis of variance was used to test for effects of treatment and also for region. Significant differences were found for both treatment ($F(1,214) = 49.49$, $P < 0.0001$) and region ($F(15,214) = 26.11$, $P < 0.0001$). The interaction was also significant ($F(15,214) = 2.74$, $P = 0.0007$). Further analysis between treatment groups in each region revealed significant differences in the majority of regions sampled (two-tailed Student's t-test with Bonferroni at a significance level set at $P = 0.05$). These are shown in figure 5.3. There was a significant increase in [^{3}H]WAY 100635 binding in the prefrontal cortex, however after Bonferroni correction only the prelimbic (PRL) and primary motor cortex (M1) subregions of the prefrontal cortex, remained significantly increased by 74.8% and 67.3% respectively. There was also a significant increase in the piriform cortex, which did not remain significant after application of the post hoc test. A significant 81.1% increase in binding to the amygdala was seen after CORT treatment. In addition, the CA1 rad, CA2 rad, CA2 pyr, CA3 rad subregions of the dorsal hippocampus sampled showed an increase in 5-HT_{1A} receptor binding after CORT treatment. But after application of Bonferroni post hoc test, only the CA1 rad and CA2 pyr subregions were found to be significantly increased by 34.8% and 80.0%, respectively. Figure 5.4 shows representative autoradiograms of brain sections from vehicle and CORT treated rats labelled with [^{3}H]WAY 100635. Alongside, are images from brain sections showing non-specific binding i.e. those which were incubated in the presence of 10 µM serotonin.

Unfortunately, it was not possible to obtain sufficient data from the PVN subregion of the hypothalamus due to the low level of specific binding in the region.

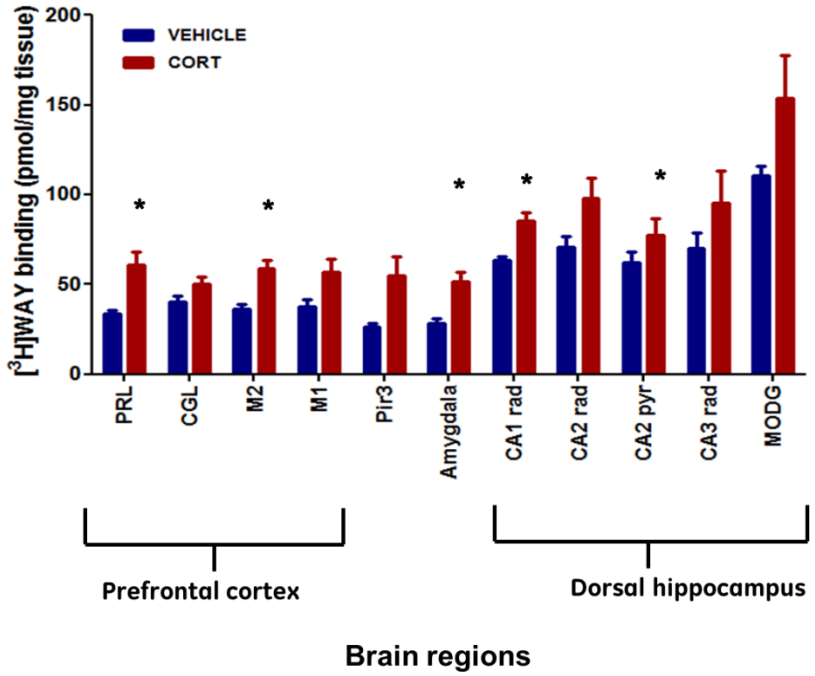


Figure 5.3. Effects of chronic corticosterone on [^{3}H]WAY 100635 binding in rat brain. Animals were treated with corticosterone in the same way as that outlined in figure 5.1. Data from regions showing a differences after corticosterone treatment are shown and expressed as mean ± SEM (n=8 per treatment group). Abbreviations for regions are the same as in figure 4.3 (chapter 4). Statistical analysis was carried out using 2-way ANOVA for treatment effects and regional effects followed by Bonferroni post hoc test for multiple comparisons, was applied with a significance level of 0.05. A statistically significant effect after correction is denoted by *.

Table 5.2 Regional [^{3}H]WAY 100635 binding in rat brain after chronic exposure to corticosterone.

Brain region	**Vehicle treated group (pmol/mg tissue)**	**CORT treated group (pmol/mg tissue)**	**% change**	**P value**
Hypothalamus				
VMDH	23.88 ± 10.56	17.32 ± 9.53	-27.5	0.308
PVN	Not detected due to high non specific binding			
Prefrontal cortex				
PrL	34.61 ± 3.03	60.51 ± 20.56*	+74.8	0.003
CgL	40.69 ± 14.96	57.32 ± 12.81	+40.9	0.032
M2	37.50 ± 6.13	62.73 ± 14.87*	+67.3	0.001
M1	37.49 ± 9.21	54.54 ± 19.78	+45.5	0.044
Piriform cortex	26.17 ± 6.91	54.84 ± 29.40	+109.6	0.017
Amygdala	28.31 ± 7.05	51.28 ± 16.47*	+81.1	0.003
Dorsal hippocampus				
CA1 rad	63.18 ± 6.44	85.15 ± 13.64*	+34.8	0.001
CA2 rad	61.94 ± 16.62	104.51 ± 30.61	+68.7	0.004
CA2 pyr	45.35 ± 7.49	81.65 ± 25.42*	+80.0	0.002
CA3 rad	55.29 ± 13.52	111.20 ± 52.44	+101.1	0.011
MODG	96.91 ± 24.57	101.30 ± 12.39	+4.5	0.659
Ventral hippocampus				
CA1 rad	93.94 ± 33.39	96.25 ± 39.52	+2.4	0.901
Subiculum	8.34 ± 2.52	12.51 ± 7.94	+0.5	0.293
Raphé	112.64 ± 19.49	103.75 ± 24.98	-7.8	0.441
Entorhinal cortex	61.31 ± 14.80	71.68 ± 16.13	+16.9	0.201

Table 5.2 Animals were treated in the same way as described in figure 6.5. Data are expressed as mean ± SD (n=8 per treatment group, except hypothalamus and subiculum where n=5 in vehicle group and n=6 in CORT group). Abbreviations used for regions are the same as in figure 4.3 (chapter 4). Statistical analysis was carried out using 2-way ANOVA for treatment effects and regional effects followed by Bonferroni post hoc test for multiple comparisons, with a significance level of 0.05. A statistically significant effect after correction is denoted by *.

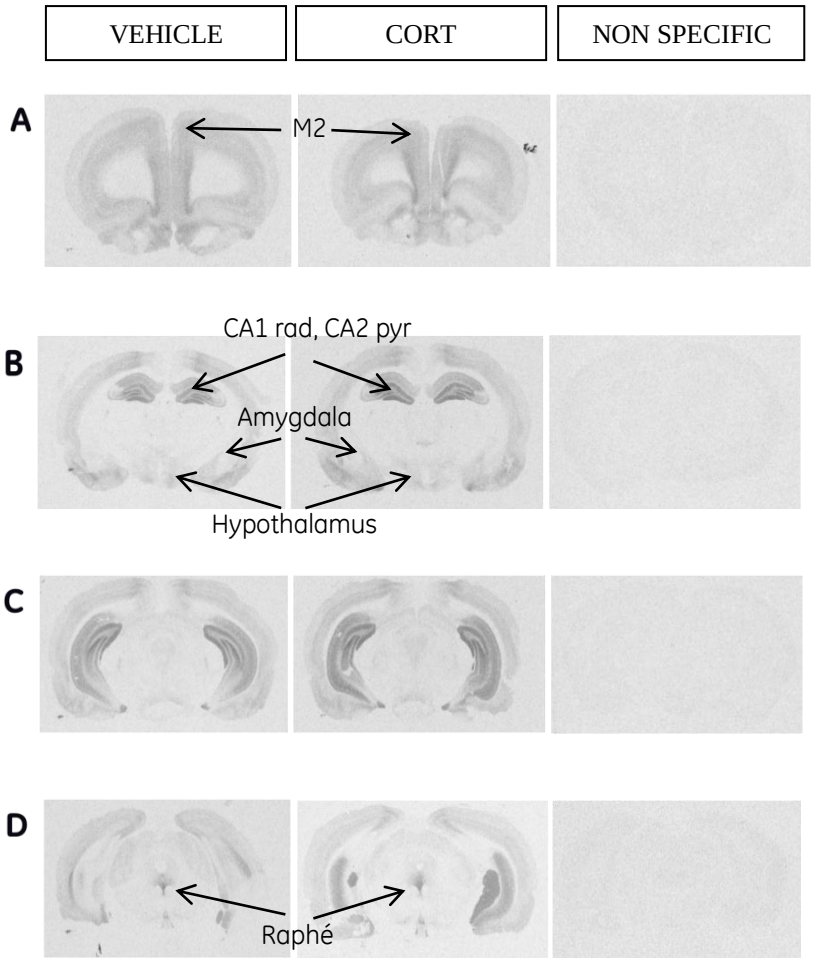


Figure 5.4 Representative [^{3}H]WAY 100635 autoradiography images from rat brain. Measured after chronic exposure to corticosterone as described in figure 6.5. Images are from individual rats representing A = prefrontal cortex (PrL, CgL, M1, M2), B = dorsal hippocampus (CA1 rad, CA2 rad, CA2 pyr, CA3 rad, MODG, amygdala, pir3, C = CA1 rad of ventral hippocampus, subiculum, D = raphé. Further details of ROI location and sampling can be found in chapter 4.

5.4.5 Indole determination by HPLC

Concentrations of 5-HT, 5-HIAA and serotonin turnover (5HIAA:5-HT) were measured in discrete brain regions after chronic exposure to CORT. Data are shown in figures 5.5 (5-HT), 5.6 (5-HIAA) and 5.7 (serotonin turnover). A two way analysis of variance was used to test for effects of CORT treatment on concentration of 5-HT, 5-HIAA and serotonin turnover measured (analysis was

carried out separately for each). No significant treatment effects were observed in any of the regions. Therefore, further analysis between treatment groups for each region was not carried out.

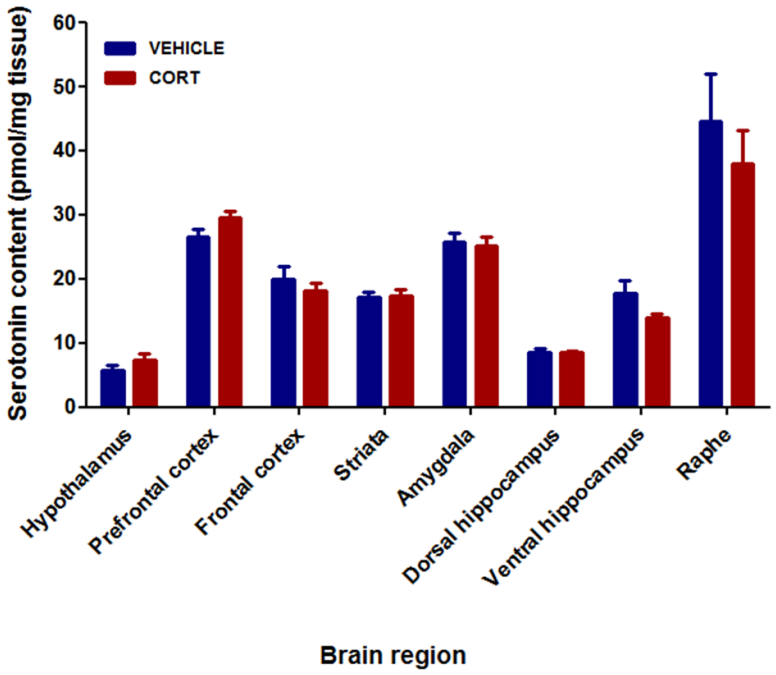


Figure 5.5. Regional serotonin content in rat brain after chronic exposure to corticosterone. Brain tissue was dissected and concentrations of 5-HT were measured using HPLC-ECD after extraction. Data are expressed as mean ± SEM (n=8 per treatment group except raphé, where n=6 per treatment group). Statistical analysis was carried out using 2-way ANOVA for treatment effects and regional effects. No statistical differences associated with corticosterone treatment were observed and therefore further statistical analysis by Student's t-test with Bonferroni correction was not carried out.

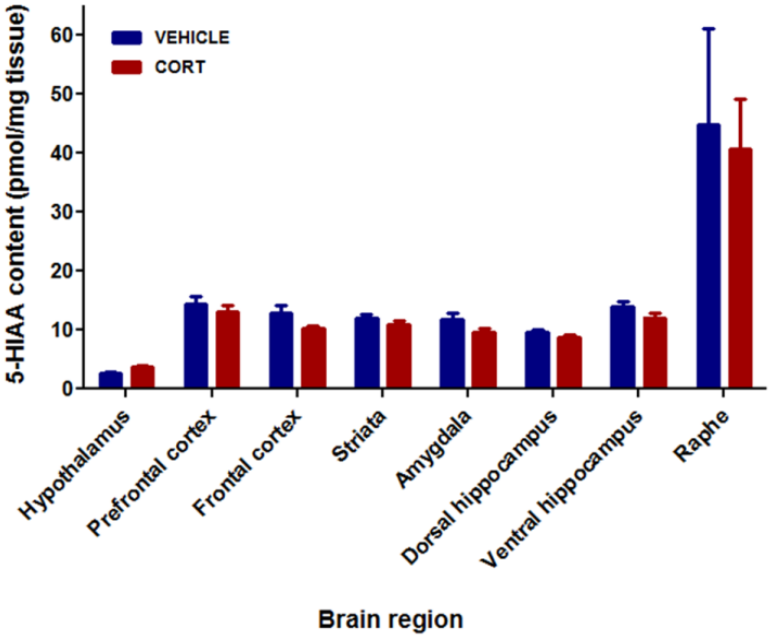


Figure 5.6. Regional 5-HIAA content in rat brain after chronic exposure to corticosterone. Brain tissue was dissected and concentrations of 5-HIAA were measured using HPLC-ECD after extraction. Data are expressed as mean ± SEM (n=8 per treatment group except raphé, where n=6 per treatment group). Statistical analysis was carried out using 2-way ANOVA for treatment effects and regional effects. No statistical differences associated with corticosterone treatment were observed and therefore further statistical analysis by Student's t-test with Bonferroni correction was not carried out.

5.5 Discussion

The present study demonstrates that long-term exposure to exogenous corticosterone attenuated body weight gain and also resulted in atrophy of the thymus and adrenal glands in male Wistar rats, consistent with suppression of the HPA axis (Magarinos et al., 1998). These are also associated with changes in the central serotonergic system. To my knowledge, this is the first report that quantifies the effects of exogenous corticosterone on 5-HT_{1A} receptor binding and serotonin tissue content under the same experimental conditions.

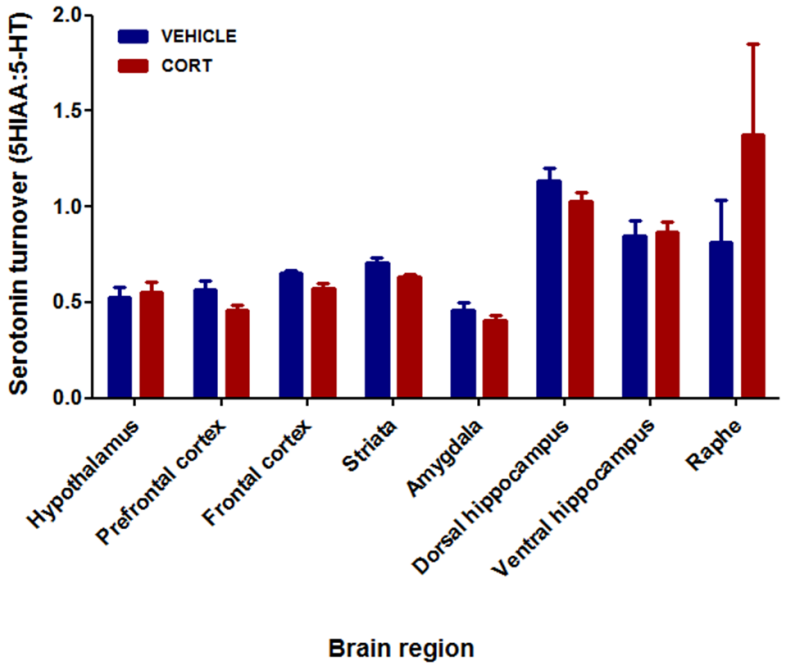


Figure 5.7 Regional serotonin turnover in rat brain after chronic exposure to corticosterone. Brain tissue concentrations of 5-HT and 5-HIAA were measured using HPLC-ECD after extraction. Serotonin turnover was calculated as the ratio of 5-HIAA:5-HT concentration. Data are expressed as mean ± SEM (n=8 per treatment group, except raphé where n=6 per treatment). Statistical analysis was carried out using 2-way ANOVA for treatment effects and regional effects. No statistical differences associated with corticosterone treatment were observed and therefore further statistical analysis by Student's t-test with Bonferroni correction was not carried out.

Corticosterone treatment affected 5-HT_{1A} receptors in brain regions implicated in responses to stress. There were no changes in the concentration of serotonin, 5-HIAA or serotonin turnover in any brain region sampled. In this section, first I discuss the effect of CORT on *in vivo* parameters such as body weight and organ weights. Then changes in 5-HT_{1A} receptor binding, indole concentration and serotonin turnover are discussed.

5.5.1 Animal study design

Male Wistar rats were selected as a stress-responsive strain, and have been extensively used in stress-related studies (Karten et al., 1999; Leventopoulos et al., 2009). The same non-invasive administration of corticosterone via drinking water (400 µg/mL for 21 days) has previously been shown to produce a flattening of the diurnal corticosterone rhythm and like the present study caused marked atrophy of the adrenal and thymus glands indicative of a suppressed HPA axis. Here I report a 48% reduction in the absolute adrenal gland weight which is comparable to the 50% reported by Donner et al. (2012). I also report a 51% reduction in thymus gland weight which is consistent with the ~54% reduction reported by Magarinos et al. (1998). In addition, and also consistent with previous work, the present CORT treatment attenuated body weight gain when compared to vehicle treated rats (Magarinos et al., 1998; Donner et al., 2012).

Although a behavioural assessment of depressive or anxiety-like behaviour was not carried out in the present study, a noticeable change in behaviour, which included reduced grooming, and increased agitation in the CORT treated animals upon handling was observed. In addition, the calculated ingested CORT dose of 55.9 mg/kg/day is comparable to previously reported behavioural studies, where daily subcutaneous injections of 40 mg/kg to rats for 21 days resulted in increased depression-like behaviour in the forced swim test with suppression of the endogenous HPA axis (Gregus et al., 2005; Johnson et al., 2006). Rats treated with exogenous CORT administration via addition to drinking water at the same dose as used here (400 µg/mL) also demonstrated

increased despair and anxiety-like behaviours in a dose dependent manner, as tested in the elevated plus maze and forced swim tests (Donner et al., 2012). Therefore, it can be assumed that the dose of CORT selected in the present study is sufficient to cause glucocorticoid related depression-like behaviour, although these were not formally investigated here.

In the present study, the volume of water normalised for body weight, revealed that CORT treated animals consumed more water than vehicle treated rats towards the end of the treatment period. This is consistent with Donner et al. (2012) where a similar trend was observed during the last week of treatment at the same dose.

5.5.2 5-HT_{1A} receptor binding

Receptor autoradiography demonstrated heterogeneous regional binding of [^{3}H]WAY 100635 consistent with the known distribution of 5-HT_{1A} receptors in rat brain (Khawaja, 1995). In the present study, exogenous CORT administration resulted in a significant and marked increase in [^{3}H]WAY 100635 binding in several brain regions involved in the regulation of mood and emotions. Specifically, 5-HT_{1A} receptor binding was significantly increased in regions such as the prelimbic (PRL) and M2 (motor cortex) subregions of the prefrontal cortex, where postsynaptic 5-HT_{1A} receptors are present. Also, CORT treatment resulted in an increase in postsynaptic receptor binding in the amygdala, as well as CA1 rad and CA2 pyr subregions of the dorsal hippocampus. However, presynaptic 5-HT_{1A} receptor binding in the raphé was unaffected by the CORT treatment. Taken together, these 5-HT_{1A} receptor

changes suggest that glucocorticoids are able to modulate 5-HT_{1A} receptors, with differential effects on pre- and postsynaptic receptors.

The presynaptic raphé 5-HT_{1A} receptors regulate serotonin release in serotonergic projection areas such as the prefrontal cortex and dorsal hippocampus and in this way, influence behavioural responses to stress by direct control over neurotransmitter release in cortical and limbic areas (Savitz et al., 2009). The present findings did not show a change in 5-HT_{1A} receptor binding in the raphé, suggesting that there was no change in the density of 5-HT_{1A} receptors in the region. However, glucocorticoids have previously been shown to affect the functionality of 5-HT_{1A} receptors in the raphé, rather than the number of 5-HT_{1A} receptors. A number of preclinical studies have indicated a desensitisation of the 5-HT_{1A} presynaptic receptors in response to chronic glucocorticoid exposure (Savitz et al., 2009). Corticosterone treatment attenuated 5-HT_{1A} receptor-mediated autoinhibitory responses to 5-HT in the raphé (Fairchild et al., 2003) and electrophysiological measurements have shown a reduction in the inhibitory action of presynaptic 5-HT_{1A} receptors in the dorsal raphé during stress (Rozeske et al., 2011). In the latter study, complementary autoradiography and Western blot studies confirmed a reduction in the function but not the expression of the 5-HT_{1A} receptor in the raphé of the same stressed animals (Rozeske et al., 2011). In addition, chronic exposure to stress did not change the number of 5-HT_{1A} receptors in the DRN, but decreased their sensitivity, an effect which was abolished by a glucocorticoid receptor antagonist, suggesting specificity to the stress response (Laaris et al., 1995; 1997).

In the present study, an increase in [^{3}H]WAY 100635 binding to the post synaptic 5-HT_{1A} receptors was observed. Significant increases were found in the

prelimbic (PRL) and motor cortex 2 (M2) subregions of the prefrontal cortex. These are important regions involved in the regulation of emotions and mood and are able to influence activity in other brain regions, coordinating the response to a stimulus (Miller and Cohen 2001). In this way, the prefrontal cortex regulates activity of raphé 5-HT neurones and hence serotonergic activity in projection regions, via a feedback mechanism between the forebrain and midbrain regions (Hajos et al., 1999, 2003; Martin –Ruiz et al., 2001). Moreover, this pathway has shown to be activated by addition of a 5-HT_{1A} receptor agonist (8-OH-DPAT) to the prefrontal cortex and can be blocked by addition of the 5-HT_{1A} receptor antagonist (WAY 100635) (Celada et al., 2001). Importantly, the reported increase in 5-HT_{1A} receptor binding in this study is consistent with the previously reported increase in [^{3}H]8-OHDPAT binding in the prefrontal cortex of tree shrews after 21 days of exposure to a psychosocial stressor, although, this was significantly reduced after 28 days (Flugge et al., 1997) suggesting time dependent effects of corticosterone on the expression of prefrontal cortex 5-HT_{1A} receptors. Taken together, it is feasible that the increased cortical 5-HT_{1A} receptor binding measured in the present study after chronic exposure to exogenous corticosterone, could represent forebrain control over serotonergic neurones in the raphé and that this increase in 5-HT_{1A} receptor binding in the prefrontal cortex may represent a transient response to elevated corticosterone levels where a possible down regulation may be seen at a later time point.

In addition, I also report an increase in [^{3}H]WAY 100635 binding in the amygdala. This region forms part of the limbic system and is involved in processing emotions such as fear and anxiety. The amygdala also modulates

memory retrieval and consolidation processes via interactions with the hippocampus and prefrontal cortex (Roozendaal et al., 2009), which are known to be impaired by high circulating levels of glucocorticoids (Roozendaal, 2002). Social stress for 20 days, which is sufficient to produce a depressed state, also resulted in an increase in amygdala 5-HT_{1A} receptor density albeit in mice (Avgustinovich et al., 2004).

The present study showed a CORT induced increase in 5-HT_{1A} receptor binding in the CA1 rad and CA2 pyr subregions. The hippocampus is the primary site for synaptic plasticity within the brain, which is mediated via the glutamate receptors (Kemp and Managhan-Vaughan 2008) and also the 5-HT_{1A} receptor (Berumen et al., 2012). More specifically, the 5-HT_{1A} receptor is involved in memory formation through long term potentiation (LTP) and associated neurogenesis via the NMDA and AMPA receptors (Berumen et al., 2012). Elevated levels of corticosterone have shown to induce changes at the AMPA and NMDA receptors which are associated with detrimental effects of stress on memory and learning (Tse et al., 2011). However, activation of the 5-HT_{1A} receptor has shown to be neuroprotective by inhibiting transport of the NMDA receptor to the dendritic surface, thereby inhibiting glutamate induced cell death (Yuen et al., 2005). Thus, the increased [^{3}H]WAY 100635 binding reported in the present study could suggest some neuroprotective effects resulting from the 5-HT_{1A} receptor upregulation although this remains speculative.

Interestingly, although I measured an increase in 5-HT_{1A} receptor binding in a number of postsynaptic regions, the majority of published studies report a decrease in 5-HT_{1A} receptor binding after exposure to glucocorticoids (Karten et

al., 1999; Czyrak et al., 2002) and an increase after adrenalectomy which is attributed to *de novo* protein synthesis (Zhong and Ciaranello, 1995). It is possible that a reduction of adrenal hormones during the 24 hour hormone free period prior to sacrifice may have resulted in an upregulation of 5-HT_{1A} receptors, similar to that seen after adrenalectomy. Although immediate effects of glucocorticoids on 5-HT_{1A} receptor mRNA have been reported (Meijer and de Kloet, 1995), it is expected that changes in receptor expression would take longer i.e. between 16-48 hours after exposure to glucocorticoids (Mendelson and McEwen 1992). Whilst the possibility that 5-HT_{1A} receptor expression in the present study may have been affected in this way cannot be completely excluded, the use of a 24 hour corticosterone free period was considered here based on previous findings that showed serotonergic responses were stable and unaffected by a 24 hour hormone period (van Gemert et al., 2006). Thus a drug free period was included in the present study in an attempt to negate any effects from an acute corticosterone exposure that would have occurred if the animals had been sacrificed immediately after the withdrawal of CORT (van Gemert et al., 2006).

Glucocorticoids are able to regulate 5-HT_{1A} receptor transcription, which occurs via both the MR and GR receptors. Low doses of glucocorticoid result in high MR occupancy and causes a reduction in 5-HT_{1A} receptor number (Chalmers et al., 1993). At high doses both MR and GR are occupied (i.e. after exposure to high doses of glucocorticoids or exposure to stress) and 5-HT_{1A} receptor binding was increased in the dorsal hippocampus (Chalmers et al., 1993). The prefrontal cortex, amygdala and dorsal hippocampus are all populated by MR and GR receptors, thereby transcriptional regulation of 5-HT_{1A}

receptors is feasible in these regions (Han et al., 2005; Roozendaal et al., 2009). This would imply that the increase in receptor binding seen here may represent an upregulation of the receptors which may be part of the GR mediated feedback mechanism on the HPA axis (Chaouloff, 1993; Flugge et al., 2004).

One final point which should also be considered is the fact that the majority of studies reported have quantified changes in 5-HT_{1A} receptor binding using the agonist radioligand [^{3}H]8-OHDPAT. This will preferentially bind to the high affinity form of the 5-HT_{1A} receptor (i.e. those receptors which are G-protein coupled) whereas in the present study an antagonist radioligand was used which binds to both high and low affinity receptor states (i.e. those receptors which are G-protein coupled and uncoupled) with equal affinity. Therefore, although the majority of preclinical studies on the effects glucocorticoids on 5-HT_{1A} receptor binding have reported a decrease in binding, the use of an agonist radioligand may not have detected the upregulation reported here if this is predominately made up of low affinity state receptors i.e. the increase in 5-HT_{1A} receptor number here could represent an attempt to 'prime' the system by increasing the receptors available for easy conversion to the high affinity site when needed.

5.5.3 5-HT and 5-HIAA content and 5-HT turnover

In order to assess whether changes in 5-HT_{1A} receptor binding were related to changes in the endogenous concentrations of 5-HT or its metabolite 5-HIAA, dissected brain tissue was analysed for indoleamine content. Many studies have shown stress related changes in 5-HT, 5-HIAA or 5-HT turnover

throughout the brain (Rueter and Jacobs 1996; Rueter et al., 1997; Maswood et al., 1998; reviewed in Flugge 2004; Amat et al., 2005) albeit with stressor and regional dependent differences having been reported (Kirby et al., 1995; Rueter and Jacobs 1996). For example, increased concentration of 5-HIAA has been reported in frontal cortex and hippocampus after forced exercise (Clement et al., 1993), whereas chronic unpredictable stress and restraint stress resulted in a decrease in 5-HIAA concentration in frontal cortex and striatum, but caused no change in hippocampus (Torres et al., 2002; Ahmad et al., 2010). In addition, exposure to stress also increased serotonin turnover in raphé, prefrontal cortex, amygdala and hippocampus (Maswood et al., 1998; Chaouloff et al., 1999; Amat et al., 2005). Interestingly, a study looking at the effects of 20 days of social stress reported no changes in 5-HT or 5-HIAA levels (Avgustinovich et al., 2004). Moreover studies looking at the effects of exogenous corticosterone on serotonergic responses are just as contradictory. A study by Inoue et al. (1996) found a decrease in 5-HT concentration and no change in 5-HIAA after an acute dose of 50 mg/kg CORT whereas after chronic exposure at the same dose there was no change in 5-HT levels, but increased 5-HIAA concentration in prefrontal cortex. The present study also showed no change in the concentration of 5-HT, 5-HIAA or serotonin turnover after chronic corticosterone administration.

There are a few possible explanations for the discrepancies in the results obtained in this study and those reported in the literature and these should be considered. The number of samples per treatment group used in the present study is small, although not much smaller than most other studies. However, it is possible that a change in either direction would have been more apparent with a larger number of animals per treatment group. In addition, here, indole levels

have been quantified in tissue homogenates, and therefore both extracellular and intracellular neurotransmitter concentration has been measured rather than that which is released in response to glucocorticoids. This may provide an explanation for the lack effect in 5-HT concentration, however serotonin turnover is considered to be sensitive enough for total tissue assessment. Also, it should be noted that as the dissection included excision of the entire region, a subregional analysis of neurotransmitter levels was not possible. Therefore the lack of effect could be due to contamination of sample from non-affected subregions. There are also major experimental differences between this study and those previously published which may impact the results. Such differences include the time of day that the study and measurements were conducted, differences in strain of animal used, animal housing and most importantly, the analytical technique i.e. microdialysis where consecutive 20 min dialysate samples representing the extracellular compartment are analysed (Chaouloff et al., 1999). Most importantly, however, it should be remembered that the brain tissue used here was obtained from animals sacrificed 24 hours after the withdrawal of CORT administered for 21 days. Thus, the measurements made here were to investigate the lasting effects of elevated glucocorticoid concentration on serotonin concentration in discrete brain regions. Typically glucocorticoids stimulate 5-HT neurones within 20-60 minutes and have reported both long lasting and rapid serotonergic effects (Luine et al., 1993; Inoue et al., 1996; Summers et al., 2000). Increases in serotonergic activity and 5-HT release have been reported during the first week of exposure to chronic stress, which returned to basal levels by the 4th week (Summers et al., 1998, Avgustinovich et al., 2004). Thus, although speculative, it is possible that any

increase in serotonin induced by the CORT treatment may have been reversed by the time of measurement at 22 days post initiation of CORT treatment. Interestingly, Karten et al. (1999) measured small changes in 5-HT neurone responses after 3 weeks of high dose CORT treatment (10 mg/rat/day; sufficient to saturate the GR for most of the day) and postulated that a resistance to the GR mediated enhancement of 5-HT develops at high doses. In the present study, the animals were ingesting 55.9 mg/kg/day, a dose that is considerably higher suggesting that it is possible that resistance of GR mediated 5-HT release may explain the lack of changes in 5-HT seen here.

Taken together, the present study showed that, CORT treatment for 21 days resulted in a significant increase in [^{3}H]WAY 100635 in the prefrontal cortex, amygdala and dorsal hippocampus without a concomitant change in serotonin concentration. This suggests that there are adaptive responses to elevated glucocorticoids, which lead to an upregulation of the 5-HT_{1A} receptor in cortical and limbic regions, which will maintain serotonergic activity. Such adaptive responses are known to occur. For example, exposure to forced swim for 15 minutes resulted in an increase in 5-HT release in the striatum but a decrease in septal nuclei of rats (Kirby and Lucki, 1997). However, the response was reversed when the same animal was exposed to a subsequent 5 min forced swim session 24 hours later, suggesting desensitisation after the initial exposure to further insult. In addition, adaptive responses in 5-HT_{1A} receptor binding have also been reported which include an upregulation or hypersensitivity of the 5-HT_{1A} receptor (Kennett et al., 1986; Flugge et al., 1997). Failure to adapt as well as HPA axis hyperactivity is thought to contribute to the aetiology of depression. Thus, the present study indicates that responses mediated via the 5-

HT_{1A} receptor in response to chronically elevated glucocorticoid concentration may be involved in such adaptive responses (McEwen, 2001).

With regards to *in vivo* clinical PET imaging studies of the 5-HT_{1A} receptor in depression, there have been conflicting results published. Both increases and decreases in binding using the PET radioligand [^{11}C]WAY 100635 have been reported (reviewed in Savitz et al., 2009). Several PET imaging studies lean towards a negative association with 5-HT_{1A} binding potential, thus reduced binding to the 5-HT_{1A} receptor has been shown in raphé (up to 42%) and hippocampus (between 23-32%) in patients with depression (Drevets et al., 1999, 2000 and 2007; Savitz et al., 2009). However, others have reported increased 5-HT_{1A} receptor binding in depression using the same radioligand. Parsey et al. (2006) reported a global increase in 5-HT_{1A} receptor binding potential in drug naïve depressed patients. However, these studies have not considered glucocorticoid dysfunction in the subjects. There is only one report of PET imaging of the 5-HT_{1A} receptor after exposure to corticosteroids (Montgomery et al., 2001). No changes in binding of [^{11}C]WAY 100635 was observed in normal patients administered an acute dose of hydrocortisone, nor was there any change after chronic exposure to prednisolone. However, it should be noted that the subjects in that study were not considered to be depressed according to the Beck Depression Inventory nor the Hamilton Depression Rating Scale. Interestingly, *post-mortem* studies on brains of human suicide victims have shown an increase in 5-HT_{1A} receptor density in the prefrontal cortex (Arango et al., 1995).

The data presented in this chapter suggest that chronic exposure to glucocorticoids leads to an upregulation of 5-HT_{1A} receptors and this may be

independent of endogenous serotonin concentration. Such receptor changes are amenable to PET imaging of depressed patients without the need to consider concomitant changes in endogenous serotonin levels.

Chapter 6

Neuropeptidergic responses to chronic glucocorticoid exposure

Oxytocin and vasopressin are both neuropeptides which regulate the activity of the HPA axis and are implicated in the aetiology of disorders associated with HPA axis dysregulation such as depression and anxiety. Preclinical studies have revealed abnormalities in both the oxytocinergic and vasopressinergic systems in response to glucocorticoid overload, effects which are mediated via the oxytocin and vasopressin 1a receptor (Ring et al., 2006; Landgraf et al., 1995). In this chapter the influence of chronically elevated glucocorticoid concentration on oxytocin and vasopressin 1a receptor binding and on the concentration of their endogenous ligands are investigated. I discuss how these changes may be related to the glucocorticoid induced dysfunction associated with stress related disorders and the serotonergic changes reported in chapter 5.

6.1 Introduction

The contribution of neuropeptides to the regulation of mood is now well established. Of particular interest are the oxytocinergic and vasopressinergic systems (Gimpl and Farenholz, 2001; Scott and Dinan, 2002). Both these peptides regulate the HPA axis, an underlying dysfunction of which has been reported in up to 50% of depressed patients (Anacker et al., 2011). Under normal conditions, oxytocin dampens HPA axis activity, having an anxiolytic effect (Heinrichs and Dome, 2008). In contrast, vasopressin acts synergistically with CRH to activate the HPA axis, resulting in an increase in glucocorticoid secretion and is therefore anxiogenic (Aguilera et al., 2007). It is thought that in depression, abnormalities in either or both the oxytocinergic and vasopressinergic regulation of the HPA axis occur, resulting in glucocorticoid dysfunction (Purba et al., 1996; van Londen et al., 1997; Scantamburlo et al., 2007). However, the exact role of the oxytocin and vasopressin receptors in the disease process is still to be elucidated. Currently there is limited information on the regulation of these receptors by glucocorticoids.

Often, in clinical studies, circulating peripheral neuropeptide concentration is measured as an indication of central oxytocinergic and vasopressinergic activity. However, the relationship between circulating levels of peptide and its release in the brain has been a matter of long-standing debate (Ebstein et al., 2012). It has been shown that both central and posterior pituitary projections originate from magnocellular hypothalamic neurones in prairie voles and rats (Young et al., 2009), which supports the idea of coupling between central and posterior pituitary release of oxytocin and vasopressin. Thus, there is an emerging tendency to accept the view that peripheral neuropeptide

concentrations are indicative of brain peptidergic activity (Shavlev et al., 2011; Ebstein et al., 2012) and could serve as a biomarker of depression. Therefore in this study, the plasma concentration of oxytocin and vasopressin was also measured after chronic exposure to corticosterone.

As already mentioned in chapter 5, administration of exogenous glucocorticoids results in neurobiological changes that are characteristic of the changes seen in depression i.e. atrophy of the hippocampus has been observed in rats receiving corticosterone in drinking water (Magarinos et al., 1998) and reduced hippocampal volume has also been reported in clinical depression (Amico et al., 2011). An enhanced understanding of the nature of the relationship between elevated glucocorticoid concentration and oxytocin and vasopressin 1a receptor binding is important. It is plausible to expect that such a relationship, if one exists, could potentially be visualised by *in vivo* molecular imaging in depression with the objective to stratify and improve the clinical management of depressed patients.

Autoradiography has previously been used to determine changes in the binding of the oxytocin receptor (Insel and Shapiro 1992; Liberzon et al., 1997) and vasopressin 1a receptor (Landgraf et al., 1995) in response to glucocorticoids in preclinical studies. The antagonist [^{125}I]OVTA (d(CH2)5Tyr(Me)2,Thr4,Orn8,[^{125}I-Tyr-NH2(9)]vasotocin), which is a selective oxytocin receptor antagonist with low cross reactivity to the vasopressin receptors has been used successfully to investigate binding to the oxytocin receptor. It has an affinity of 0.1 nM for the oxytocin receptor (Liberzon and Young, 1997). A high density of binding was observed in the neocortex, limbic system and hypothalamus (Klein et al., 1995). For the vasopressin 1a receptor,

the antagonist, [^{125}I]AVP ([phenylacetyl 1,0-ME-D-tyr 2 ARG 6,8,TYR 9] ^{125}I), which is a selective antagonist at the vasopressin 1a receptor with an affinity of 1.6 nM has previously been reported to label the receptors (Thibonnier et al., 2000). [^{125}I]AVP demonstrates a high level of binding in the lateral septum, BNST, nucleus accumbens, central nucleus of amygdala, hippocampus, thalamus, and superior colliculus of rat brain (Phillips et al., 1988).

In this chapter, I describe the experimental studies undertaken to determine changes in oxytocin receptor and V1aR binding after exposure to exogenous glucocorticoids. In addition, the regional brain content of each neuropeptide was measured using RIA. Peripheral (plasma) oxytocin and vasopressin concentrations were also measured to compare them with those of brain tissue neuropeptides. This is of interest given the opposing roles of oxytocin and vasopressin on regulation of the HPA axis; the latter being anxiogenic and the former anxiolytic. Oxytocin governs emotional behaviours that lead to positive social interactions (Heinrichs et al., 2003; Kosfeld et al., 2005; Debiec 2007) and is released in the hippocampus, amygdala, septum and hypothalamus i.e. regions that are rich in glucocorticoid receptors and involved in the regulation of mood (Engelmann et al., 1996; Liberzon and Young 1997 Huber et al., 2005). The effect of chronically elevated glucocorticoids on vasopressin 1a receptor binding in extralimbic brain regions has not previously been studied, although interactions between the vasopressinergic and serotonergic system are established. It has been shown that the 5-HT_{1A} receptor antagonist WAY100635 inhibits 5-HT induced release of oxytocin and vasopressin (Jørgensen et al., 2003). Whereas, selective serotonin inhibitors (SSRIs) are known to induce the release of oxytocin (Uvnas Moberg et al.,

1999). The V1a receptor is of relevance to aggression-related behaviour (McCall and Singer, 2012). Aggression is thought to be regulated via interactions between the serotonin and vasopressin systems, whereby serotonergic synapses have been observed on vasopressinergic neurones at the level of the hypothalamus, with serotonergic innervation to the hypothalamus originating from the raphé (Ferris and Delville, 1999). Thus, it is of interest to determine the vasopressinergic response to glucocorticoids in relation to those of the serotonergic and oxytocinergic systems.

There are several aspects of novelty to this study, to my knowledge this is the first report in which changes at both oxytocin receptor and vasopressin 1a receptor have been measured in both limbic and extralimbic regions of the same tissue in response to chronic exposure to exogenous glucocorticoids. In addition, this is the first time that changes in oxytocin and vasopressin 1a receptor binding have been related to concentrations of their endogenous ligands.

6.1.1 Oxytocin and the stress response

Oxytocin is synthesised in the hypothalamic paraventricular (PVN) and supraoptic nuclei (SON) and released to blood in the posterior pituitary. Oxytocin is also released somatodendritically within the hypothalamic nuclei; from where it can diffuse to other brain regions (Leng and Ludwig, 2008). Thus oxytocin is present in the hippocampus, amygdala, septum and hypothalamus. These brain regions also express oxytocin receptors (Leng and Ludwig, 2008) and glucocorticoid receptors; (Engelmann et al., 1996; Liberzon and Young 1997; Huber et al., 2005). Therefore a role for interactions between

glucocorticoids and oxytocin in the regulation of the responses to stress is postulated.

Chronic administration of oxytocin to rats transiently activates the HPA axis and results in an increase of corticosterone release, followed by suppression of the HPA axis (Petersson et al., 1999). More specifically, evidence shows that oxytocin is anxiolytic via inhibition of the HPA axis, which leads to the lowering of glucocorticoid concentration (Neumann et al., 2000; Heinrichs and Dome, 2008). Intracerebral administration of the neuropeptide has been shown to also counteract anxiety–like behaviours measured as reduced immobility during the forced swim test and reduced vocalisation of distress in rodent models (Arletti and Bertolini, 1987; Insel and Winslow, 1991; Windle et al., 1997).

The central effects of oxytocin are mediated through the oxytocin receptor and this is responsible for the anxiolytic effects of the peptide. Pharmacological doses (10µg i.c.v. or 30mg/kg i.p.) of oxytocin induce anxiolytic effects in preclinical models of anxiety (elevated maze test). These effects can be blocked by the addition of an oxytocin receptor antagonist suggesting the anxiolytic effects of oxytocin are mediated by direct action at the oxytocin receptor (Ring et al., 2006; Yoshida et al., 2009). Furthermore, administration of an oxytocin receptor antagonist increased stress-induced corticosterone release in rats suggesting that the oxytocin receptor is directly involved in mediating the responses to stress (Neumann et al., 2000). In addition, oxytocin receptor knockout mice show deficits in social behaviour and an increase in aggression-like behaviour suggesting that disruptions at the oxytocin receptor could potentially result in altered social behaviours, as seen in mood disorders (Takayanagi et al., 2005). Moreover, behaviours which are associated

with reduced anxiety, such as lactation, cause a release of oxytocin in the brain which is positively correlated with oxytocin receptor binding in the lateral septum of rats (Curly et al., 2012). Whereas, anxiety behaviours, measured as activity in the open field test of normal animals are negatively correlated with oxytocin receptor binding. Thus, it has been suggested that the oxytocin receptor is involved in mediating the anxiolytic effects of oxytocin (Curly et al., 2012).

In humans, intranasal administered oxytocin initiates prosocial behaviours such as approach, social interaction, trust and risk-taking, all of which are associated with reduced cortisol production (Heinrich et al., 2003; Kosfeld et al., 2005). In addition, depressed patients tend to have reduced plasma oxytocin concentrations when compared to age-matched controls (Frasche et al., 1995 and Scantamburlo et al., 2007), although elevated oxytocin activity has also been reported in depression (Parker et al., 2010; Holt-Lunstad et al., 2011). These discrepancies may reflect the complexity of depression as a multi-factorial condition and an uncertain link between peripheral and central oxytocin. Analysis of *post mortem* brains from depressed patients has shown a 23% increase in the number of oxytocinergic immunoreactive neurones in the PVN. However, a more detailed attempt in the same study to use this to distinguish major depression from bipolar depression in this group failed to find any differences between these subtypes of depression (Purba et al., 1996).

6.1.2 Vasopressin and the stress response

Vasopressin is also released from the SON in a similar way to oxytocin into both the general circulation and centrally. Parvocellular neurones of the PVN, amygdala and BNST also release AVP (DeVries et al., 1985). In contrast

to oxytocin, vasopressin is anxiogenic and aggression promoting via activation of the HPA axis, particularly during chronic stress (Aguilera et al., 2007). Chronic stress increases the expression of vasopressin in the PVN and its secretion into the pituitary portal circulation, stimulating the HPA axis (Van de Kar and Blair, 1999). This effect was consistent and irrespective of the type of stressor. For example forced swimming in rodents produces an increase in AVP in the PVN and SON (Wotjak et al., 1998) and also in the septal nuclei and amygdala, but this was prevented when the V1aR antagonist d(Ch2)5Tyr(Me)AVP was administered in the lateral and medial septal nuclei (Ebner et al., 1999; 2002). Also, training in the Morris water maze for three days also caused a release of AVP in these regions (Engelmann et al., 2006). In addition, anxiety-like behaviour as measured by the resident-intruder test is associated with AVP release into the septal nuclei (Veenema and Neumann, 2008).

The vasopressin V1a receptor is implicated in mediating the anxiogenic effects of AVP as shown by the studies of Ebner et al. (1999; 2002) mentioned above. V1aR overexpression is associated with increased anxiety as measured by the time spent in the open arm of the elevated plus maze test and is reversed by a V1aR antagonist (Landgraf et al., 1995).

In humans, AVP is associated with depression where patients show significantly elevated plasma AVP concentration (van Londen et al., 1997). Those depressed patients who progressed to attempt or commit suicide showed even higher concentration of plasma AVP and vasopressin immunoreactivity in the PVN, suggesting that vasopressin may be linked with severity of the disorder (Inder et al., 1997; Merali et al., 2006). Furthermore, vasopressin is involved in

the onset and maintenance of a high anxiety state and its concentration in plasma has been shown to correlate both with anxiety and anxiety-related retardation in depression, activation of the HPA axis and in a rise in salivary cortisol, which is further increased during a social evaluative threat (Shalev et al., 2011). In addition, a *post mortem* study showed increased activation of oxytocin and vasopressin neurones in the paraventricular nucleus of depressed subjects, as measured by an increase in vasopressin immunoreactivity (Purba et al., 1996).

7.2 Aims and Hypotheses

7.2.1 Aims

This thesis investigates if long-term exposure to exogenous glucocorticoids (corticosterone) affects oxytocin and V1a vasopressin receptor binding in rat brain post-mortem and whether the changes are associated with regional oxytocin and vasopressin content. This is the first study to investigate the role of both of these peptide receptors in responses to glucocorticoids. It can be of translational value in the development of future pharmacological tools to manipulate the receptor activity and/or visualise it *in vivo*.

As outlined above, oxytocin is a contributor to the stress response as this is released into the bloodstream in response to a stressful stimulus (Neumann et al., 2000). Furthermore, the oxytocin and 5-HT system are interconnected through oxytocin fibres which project into the raphé, where serotonin and oxytocin may influence each other. Therefore, it is conceivable that increased corticosterone levels may also impact on oxytocin.

In order to ensure homeostatic control, a negative feedback mechanism (HPA re-activity) is initiated. It has been found that oxytocin released at the

same time as CRH will potentiate HPA re-activity and enforce the negative feedback mechanism. However, a study by Neumann et al., 2000 found that in the presence of an oxytocin receptor antagonist, the negative feedback mechanism prevails. It is suggested that oxytocin may activate CRH neurones directly producing an even greater increase in CRH which would induce HPA re-activity, thus in this way oxytocin serves to protect the damage associated with excess corticosterone production. In the present study, brain regional oxytocin receptor binding as well as oxytocin content is quantitatively analysed in order to establish the role of the central oxytocin system in response to glucocorticoids.

On the other hand and as mentioned above, vasopressin activates the HPA axis; in particular vasopressin is released during stress and is the main activator of the HPA axis (Scott and Dinan, 1998). The effect of glucocorticoids on vasopressin 1a receptor binding in extralimbic brain regions has not previously been studied, nor have changes in receptor binding after glucocorticoid exposure been associated with vasopressin concentration, therefore this study aims to establish this relationship.

7.2.2 Hypothesis

It is hypothesised that chronic exposure to glucocorticoids will result in changes to the oxytocin and vasopressin 1a receptors in brain regions that are involved in the regulation of responses to stress. The direction of changes in receptor binding will reflect changes in the concentration of their endogenous neurotransmitter. In addition, it is hypothesised that plasma concentrations of

oxytocin and vasopressin will reflect central concentrations of each neuropeptide.

6.3 Materials and Methods

All materials used for this study are given in chapter 4. Also, in chapter 4, are methodological details on *in vivo* husbandry, dosing on animals, collection of post mortem samples and tissue sectioning, autoradiography and RIA methodology.

It should be noted that the autoradiography study was carried out in animals that were given a 24 hour hormone free period before sacrifice (under light anaesthesia), whereas oxytocin and vasopressin content was determined in animals that were sacrificed without this hormone free period and without the use of anaesthetic. In addition, plasma corticosterone was also determined from this subset and is shown alongside the plasma corticosterone content of animals with the 24 hour drug free period (as two timepoints 0hrs and 24hrs).

6.4 Results

The autoradiography part of this study used tissue generated in the study presented in chapter 5. Changes in body weight, water consumption, adrenal and thymus gland weights as well as the calculated CORT dose received by these animals is therefore as presented in chapter 5. For the additional *in vivo* dosing experiments conducted in this study i.e. those for neuropeptide RIA experiments a two-way analysis of variance was used to test for differences in weight gain,

water consumption and also adrenal and thymus gland weights. No significant differences were observed in any parameter measured. Therefore, in this chapter, the two batches of animals have been considered as a single group with two timepoints; 0hr (n=8 for vehicle group and n=10 for CORT group) and 24hr (n=8 per treatment group), representing animals sacrificed immediately after withdrawal of CORT and those sacrificed after a 24 hour hormone free period respectively. The statistical comparison can be found in appendix B.

6.4.1 Plasma corticosterone concentration

Concentration of plasma corticosterone in vehicle and CORT treated groups was measured using RIA at both 0hr and 24hr timepoints. Data are shown in figure 6.1. A two way analysis of variance was used to test for effects of treatment on the plasma concentration of corticosterone and also any effects associated with timepoint of sample. Significant differences were found for treatment ($F(1,41) = 8.78$, $P = 0.0051$) and timepoint ($F(1,41) = 28.22$, $P = <0.0001$). The interaction was also significant ($F(1,41) = 28.04$, $P = <0.0001$). Further analysis between treatment groups at individual time points (vehicle vs CORT at 0hr and 24hr) was carried out using the two-tailed Student's t-test (unpaired) with Bonferroni correction at a significance level of $P = 0.05$. This revealed that there was a significant 282.4% increase in plasma corticosterone concentration of the CORT treated group when compared to the vehicle treated group at t=0hrs (from 109.6 ± 100.1 ng/mL to 419.1 ± 249.6 ng/mL). Whereas in the 24hr group, plasma corticosterone concentration was significantly reduced by 80.2% in the CORT treated group (from 109.0 ± 43.9 ng/mL to 21.5 ± 15.0 ng/mL) indicative of HPA axis suppression. In addition, two-tailed Student's t-

test (unpaired) with Bonferroni correction was used to test for differences within each treatment group at individual time points (vehicle treated at 0hr and 24hr; and CORT treated at 0hr and 24hr) with a significance level set at P = 0.05. This showed that there was no significant difference in plasma corticosterone at 0hr and 24hr in the vehicle treated animals (109.6 ± 100.05 ng/mL and 109.0 ± 43.93 ng/mL respectively). However, there was a significant 94.8% decrease in plasma corticosterone concentration in the CORT treated group at 24hrs when compared to 0hrs (from 419.1 ± 249.6 ng/mL at 0hrs to 21.54 ± 14.99 ng/mL at 24hrs) (Figure 6.1).

6.4.2 Autoradiography

6.4.2.1 Oxytocin receptor binding

Changes in binding to the oxytocin receptor were quantified after chronic exposure to CORT. The quantitative data showing [^{125}I]OVTA specific binding to oxytocin receptors are shown in table 6.1. A two way analysis of variance was used to test for effects of treatment and also for region. Significant differences were found for both treatment ($F(1,114) = 20.74$, $P = <0.0001$) and region. ($F(8,114) = 71.35$, $P = <0.0001$). The interaction was also significant ($F(8,114) = 6.07$, $P = <0.0001$). Further analysis between treatment groups (veh vs CORT) in each region using two-tailed Student's t-test (unpaired) with Bonferroni correction with a significance level of P = 0.05 revealed significant differences in many regions.

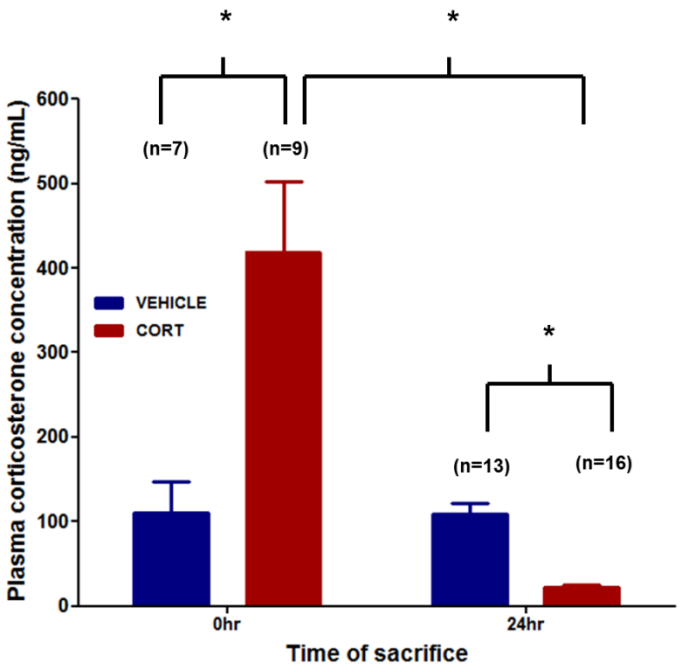


Figure 6.1. Plasma corticosterone level determined by RIA. Animals were treated with either 400 µg/mL CORT or vehicle for 21 days. Data are expressed as mean ± SEM. Statistical analysis was carried out using two-way ANOVA for effect of treatment and timepoint. Note: A total of 5 data points were removed from the final analysis due to technical errors during the pipetting of samples which meant that results obtained were inaccurate and outside the range of calibration standards

These are shown in figure 6.2. Specifically, there was a significant 60.0% decrease in [^{125}I]OVTA binding in the hypothalamus. However, binding was significantly increased in the LSD subregion of the septal nuclei by 87.5% and also in the raphé by 100%. In addition, there was a tendency towards an increase in [^{125}I]OVTA binding in the CeA and subiculum ($P<0.1$). There was no change in oxytocin receptor binding in the whole dorsal hippocampus or insular cortex. It should be noted that data from the ventral hippocampus is from a total of 5 vehicle treated animals and 4 CORT treated animals rather than 8 as for the other regions. The variation in the sample size is due to a tearing of the tissue in the subiculum region, which meant that accurate sampling was not possible. Unfortunately, it was not possible to obtain sufficient data from the PVN

subregion of the hypothalamus and prefrontal cortex subregions due to the high non specific binding of the radioligand. Figure 6.3 shows representative autoradiograms of brain sections from vehicle and CORT treated rats labelled with [^{125}I]OVTA. Alongside, are images from brain sections showing non-specific binding i.e. those which were incubated in the presence of 100 µM oxytocin.

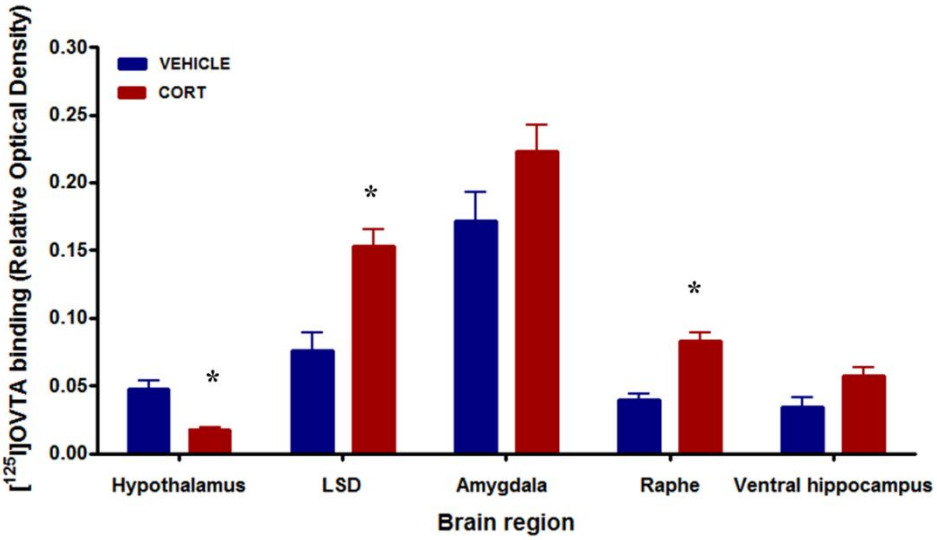


Figure 6.2. Effects of chronic corticosterone on [^{125}I]OVTA binding in rat brain. Animals were treated with corticosterone in the same way as that outlined in figure 6.1. Data from regions showing a differences or tendency towards change after corticosterone treatment are shown and expressed as mean ± SEM (n=8 per treatment group, except ventral hippocampus where n=6 for vehicle group and n=5 for CORT treated group). Abbreviations are the same as in figure 4.4, chapter 4. Statistical analysis was carried out using 2-way ANOVA for treatment effects and regional effects followed by a Bonferroni post hoc test for multiple comparisons, with a significance level of 0.05. A statistically significant effect after correction is denoted by *.

Table 6.1. Regional [^{125}I]OVTA binding to oxytocin receptors in rat brain after chronic exposure to corticosterone.

Brain region	**Vehicle treated group (ROD)**	**CORT treated group (ROD)**	**% change**	**P value**
Hypothalamus				
VMDH	0.05 ± 0.02	0.02 ± 0.005 *	-60.0	0.0002
PVN	Not detected due to high non specific binding of radioligand			
Prefrontal cortex				
PrL				
CgL	Not detected due to high non specific binding of radioligand			
M1				
M2				
Septal Nuclei				
LSD	0.08 ± 0.04	0.15 ± 0.04 *	+87.5	0.0008
MSD	0.04 ± 0.01	0.05 ± 0.01	+25.0	0.4906
Amygdala				
CeA	0.17 ± 0.06	0.22 ± 0.05	+29.4	0.0962
Dorsal hippocampus	0.03 ± 0.01	0.03 ± 0.01	0.0	0.6119
Ventral hippocampus *Subiculum*	0.03 ± 0.02	0.06 ± 0.01	+100	0.0924
Raphé	0.04 ± 0.01	0.08 ± 0.02 *	+100	<0.0001
Insular Cortex	0.03 ± 0.01	0.03 ± 0.01	0.0	0.9729

Table 6.1 Animals were treated in the same way as described in figure 6.1. Data are expressed as mean ± SD (n=8 per treatment group, except ventral hippocampus where n=5 for vehicle group and n=4 for CORT treated group). Abbreviations used for regions are the same as in figure 4.4, chapter 4. Statistical analysis was carried out using 2-way ANOVA for treatment effects and regional effects followed by a Bonferroni post hoc test for multiple comparisons, with a significance level of 0.05. A statistically significant effect after Bonferroni correction is denoted by *.

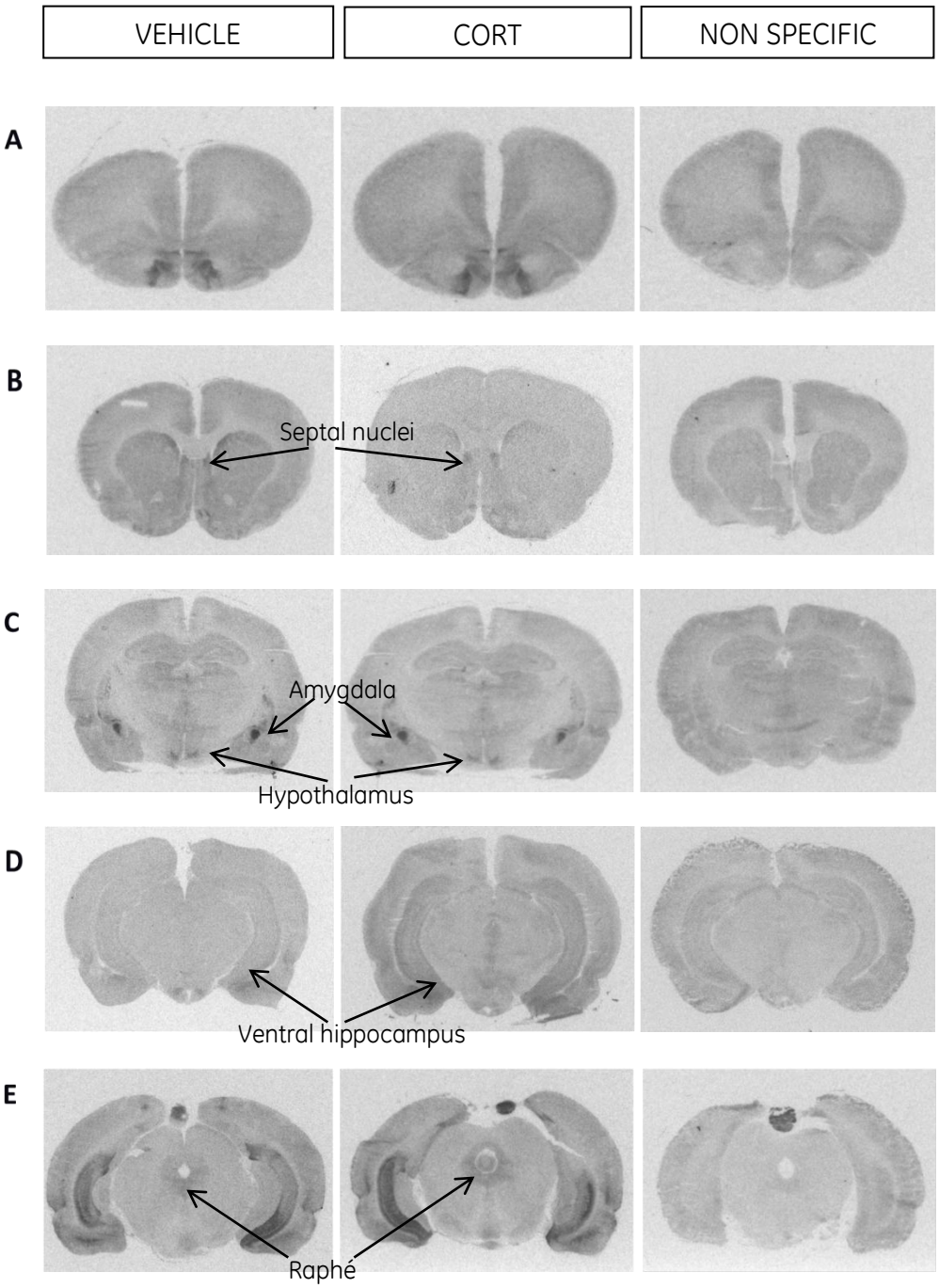


Figure 6.3 Representative [^{125}I]OVTA autoradiography images from rat brain. Measured after chronic exposure to corticosterone as described in figure 6.1. Images are from individual rats representing A = prefrontal cortex, B = septal nuclei (LSD, MSD), insular cortex, C = dorsal hippocampus, amygdala, hypothalamus, D = ventral hippocampus, E = raphé. Further details of ROI location and sampling can be found in chapter 4.

6.4.2.2 Vasopressin receptor binding

Changes in binding to the vasopressin 1a receptor were quantified after chronic exposure to CORT. The data representing [^{125}I]AVP specific binding to vasopressin 1a receptors are shown in table 6.2. A two way analysis of variance was used to test for effects of treatment and also for region. Significant differences were found for both treatment ($F(1,168) = 14.05$, $P = 0.0002$) and region ($F(11,168) = 14.46$, $P = <0.0001$). The interaction was also significant ($F(11,168) = 3.19$, $P = 0.0006$). Further analysis between treatment groups in each region using two-tailed Student's t-test (unpaired) with a Bonferroni post hoc test at a significance level of $P = 0.05$ revealed significant differences in some regions. These are shown in figure 6.4. There was a significant 42.1% decrease in [^{125}I]AVP binding in the VMDH. Binding was increased in all other brain regions sampled, reaching significance in the LSD subregion of the septal nuclei and PODG subregion of the dorsal hippocampus where [^{125}I]AVP binding was increased by 30.0% and 78.6% respectively. In addition, a significant increase in binding to the vasopressin 1a receptor was measured in the raphé, but significance was lost after correction for multiple comparisons with the Bonferroni post hoc test. Unfortunately, it was not possible to obtain sufficient data from the PVN subregion of the hypothalamus, prefrontal cortex subregions and subiculum due to the high non specific binding of the radioligand. Figure 6.5 shows representative autoradiograms of brain sections from vehicle and CORT treated rats labelled with [^{125}I]AVP. Alongside, are images from brain sections showing non-specific binding i.e. those which were incubated in the presence of 50µM vasopressin.

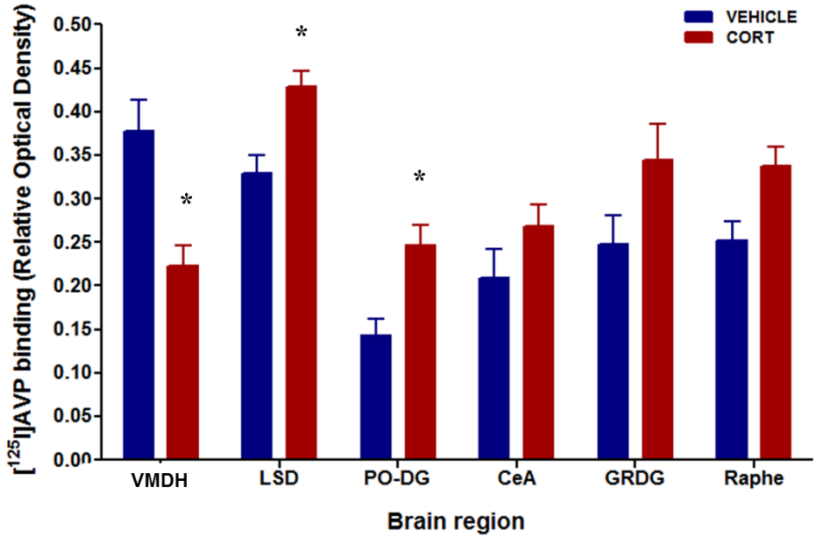


Figure 6.4. Effects of chronic corticosterone on [^{125}I]AVP binding in rat brain. Animals were treated with corticosterone in the same way as that outlined in figure 6.1. Data from regions showing a differences after corticosterone treatment are shown and expressed as mean ± SEM (n=8 per treatment group). Abbreviations for regions are the same as in figure 4.4, chapter 4. Statistical analysis was carried out using 2-way ANOVA for treatment effects and regional effects followed by a Bonferroni post hoc test for multiple comparisons, applied with a significance level of 0.05. A statistically significant effect after correction is denoted by *.

Table 6.2. Regional [^{125}I]AVP binding to vasopressin 1a receptors in rat brain after chronic exposure to corticosterone.

Brain region	Vehicle treated group (ROD)	CORT treated group (ROD)	% change	P value
Hypothalamus				
VMDH	0.38 ± 0.10	0.22 ± 0.07*	-42.1	0.0034
PEFLH	0.21 ± 0.09	0.23 ± 0.07	+9.5	0.6572
PVN	Not detected due to high non specific binding of radioligand			
Prefrontal cortex				
PrL				
CgL	Not detected due to high non specific binding of radioligand			
M2				
M1				
Septal Nuclei				
LSD	0.33 ± 0.06	0.43 ± 0.05*	+30.0	0.0026
Amygdala				
CeA	0.21 ± 0.09	0.27 ± 0.07	+28.6	0.1742
Dorsal hippocampus				
PODG	0.14 ± 0.05	0.25 ± 0.06*	+78.6	0.0036
MODG	0.16 ± 0.06	0.19 ± 0.04	+18.7	0.1738
Ventral hippocampus				
GRDG	0.25 ± 0.10	0.34 ± 0.12	+36.0	0.0964
Subiculum	Not detected due to high non specific binding of radioligand			
Raphé	0.25 ± 0.06	0.34 ± 0.06	+36.0	0.0140
Nucleus Accumbens				
Shell	0.39 ± 0.09	0.45 ± 0.08	+15.4	0.1560
Thalamus				
PO	0.19 ± 0.07	0.25 ± 0.09	+31.6	0.1603
LDDM	0.29 ± 0.10	0.31 ± 0.11	+6.9	0.6717
VM	0.21 ± 0.07	0.22 ± 0.04	+4.8	0.5727

Table 6.2 Animals were treated in the same way as described in figure 6.1. Data are expressed as mean ± SD (n=8 per treatment group). Abbreviations used for regions are the same as in figure 4.4, chapter 4. Statistical analysis was carried out using 2-way ANOVA for treatment and regional effects followed by a Bonferroni post hoc test for multiple comparisons, at a significance level of 0.05. A statistically significant effect after Bonferroni correction is denoted by *.

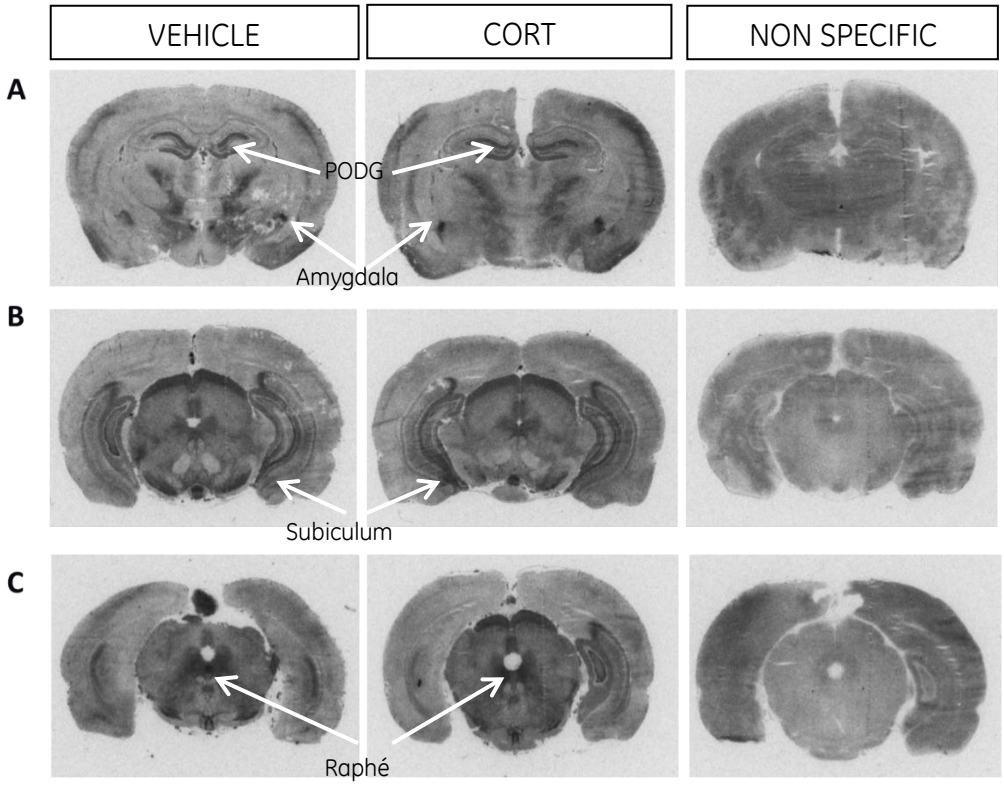


Figure 6.5 Representative [^{125}I]AVP autoradiography images from rat brain. Measured after chronic exposure to corticosterone as described in figure 6.1.Images are from individual rats representing A = dorsal hippocampus, (PODG, MODG), thalamus (PO, LDDM, VM), amygdala, hypothalamus (VMDH, PEFLH) B = ventral hippocampus (subiculum), C = raphé. Further details of ROI location and sampling can be found in chapter 4.

6.4.3 Neuropeptide concentration

The concentrations of oxytocin and vasopressin were measured in discrete brain regions and plasma (0hr) after chronic exposure to CORT. Data describing oxytocin concentration is shown in table 6.3 and vasopressin concentration in table 6.4. A two way analysis of variance was used to test for effects of CORT treatment on the concentration of each neuropeptide and also for any regional effects. Separate analyses were conducted for oxytocin and vasopressin.

With regards to oxytocin concentration, significant differences were found for both treatment ($F(1,94) = 16.99$, $P = <0.0001$) and region ($F(7,94) = 52.94$, $P = <0.0001$). The interaction was also significant ($F(7,94) = 16.43$. $P = <0.0001$). Further analysis between treatment groups in each region was carried out using two-tailed Student's t-test (unpaired) with Bonferroni correction at a significance level set of $P = 0.05$ which revealed that there was a significant 230.8% increase in oxytocin concentration in the hypothalamus. This was the only brain region where oxytocin concentration was significantly affected by CORT treatment. In addition, although a 40.5% decrease in plasma oxytocin concentration was seen, but this did not reach significance.

Analysis of brain vasopressin content did not reveal any significant effect of CORT treatment on vasopressin concentration in any region sampled. For this reason, further analysis between treatment groups for each region was not carried out. In the plasma, vasopressin concentration was only increased by 9.3% (again not significant).

An accurate analysis of vasopressin in hypothalamus and oxytocin in pituitary gland could not be determined as the levels were above the calibration range of the assay (1280 pg/mL). An attempt was made to further dilute the extracts (1:100) and re-run the test, however these remained above the range of the calibration curve. Unfortunately, there was a large variation in the number of data points in each group ranging between 5-10, out of a possible n=8 in the vehicle treated group and n=10 in the CORT treated group. These were data points that fell outside of the calibration curve due to experimental errors and were removed from the analysis. In addition, outliers were removed from the

data, defined as those with a value greater than 2 standard deviations from the mean. The n numbers for each are given alongside the data in tables 6.3 and 6.4

Table 6.3 Oxytocin content in discrete brain regions and plasma

Tissue	Oxytocin content (pg/mg tissue) Vehicle group	Oxytocin content (pg/mg tissue) CORT group	% change	p value
Pituitary gland	*Above 1280 pg/mL*	*Above 1280 pg/mL*	-	-
Hypothalamus	57.83 ± 42.37 (n=6)	191.3 ± 70.34 * (n=7)	+230.8	0.002
Prefrontal cortex	0.63 ± 0.40 (n=7)	0.73 ± 0.70 (n=7)	+15.9	0.760
Septal nuclei	8.68 ± 4.57 (n=7)	11.55 ± 6.23 (n=8)	+33.1	0.335
Amygdala	3.44 ± 3.29 (n=6)	2.12 ± 1.31 (n=7)	-38.4	0.350
Dorsal hippocampus	0.22 ± 0.18 (n=5)	0.36 ± 0.28 (n=8)	+63.6	0.349
Ventral hippocampus	0.58 ± 0.30 (n=6)	0.34 ± 0.25 (n=9)	-41.4	0.126
Raphé	8.18 ± 5.21 (n=6)	13.76 ± 14.05 (n=7)	+68.2	0.379
Plasma (ng/mL)	16.83 ± 12.42 (n=7)	10.01 ± 6.51 (n=6)	-40.52	0.254

Table 6.3. Effects of chronic corticosterone on oxytocin concentration in discrete rat brain regions and plasma. Animals were treated with corticosterone in the same way as that outlined in figure 6.1. Data are shown as mean ± SD (n=5-9 per treatment group). Statistical analysis was carried out using 2-way ANOVA for treatment effects and regional effects followed by a Bonferroni post hoc test for multiple comparisons, with a significance level set at 0.05. A statistically significant effect after correction is denoted by *.

Table 6.4 Vasopressin content in discrete brain regions

Tissue	Vasopressin content (pg/mg tissue) Vehicle group	Vasopressin content (pg/mg tissue) CORT group	% change	p value
Pituitary gland	53.35 ± 23.57 (n=8)	50.08 ± 20.50 (n=8)	-6.1	0.771
Hypothalamus	*Above 1280 pg/mL*	*Above 1280 pg/mL*	-	-
Prefrontal cortex	1.34 ± 0.75 (n=7)	1.24 ± 0.61 (n=10)	-7.5	0.756
Septal nuclei	9.43 ± 5.48 (n=6)	11.95 ± 4.66 (n=7)	+26.7	0.390
Amygdala	5.09 ± 2.70 (n=6)	9.30 ± 4.31 (n=9)	+82.7	0.054
Dorsal hippocampus	2.06 ± 1.90 (n=6)	1.58 ± 0.94 (n=10)	-23.3	0.505
Ventral hippocampus	3.67 ± 3.18 (n=8)	2.17 ± 1.03 (n=10)	-40.9	0.175
Raphé	68.27 ± 53.64 (n=7)	40.92 ± 30.08 (n=7)	-40.0	0.262
Plasma (ng/mL)	172.51 ± 33.56 (n=7)	188.88 ± 26.97 (n=10)	+9.5	0.282

Table 6.4 Effects of chronic corticosterone on oxytocin concentration in discrete rat brain regions. Animals were treated with corticosterone in the same was as that outlined in figure 6.1. Data are shown as mean ± SD (n=6-10 per treatment group). Statistical analysis was carried out using 2-way ANOVA for treatment effects and regional effects. No statistical differences associated with corticosterone treatment were observed and therefore further statistical analysis by Student's t-test with Bonferroni correction was not carried out.

In an attempt to assess the relationship between the concentration of plasma neuropeptide and of those measured in regional brain tissue, a correlation analysis was carried out between plasma and brain neuropeptide concentration measured. There was no significant correlation between plasma and tissue oxytocin/vasopressin levels in either treatment group. Neither was there any correlation between plasma corticosterone and plasma or tissue neuropeptide concentration. The correlation coefficient values are presented in appendix C.

6.5 Discussion

The present study demonstrates that chronic exposure to glucocorticoids resulted in changes within the central oxytocinergic and vasopressinergic systems. This is the first study to describe the direct influence of glucocorticoids on both oxytocin and vasopressin activity in the same material and also to this extent throughout the rat brain. Chronic treatment with corticosterone resulted in changes in oxytocin and vasopressin 1a receptor binding in the brain regions that are involved in the regulation responses to stress. In addition, there was a significant increase in the oxytocin content of the hypothalamus after CORT treatment. No significant changes were observed in the vasopressin content of any brain region sampled. Likewise, there were no significant changes in plasma concentrations of either peptide in response to chronic CORT exposure.

6.5.1 Plasma corticosterone concentration

Plasma concentrations of corticosterone were significantly increased in CORT treated animals that were sacrificed at t=0 hr. This is in line with the previous reports where plasma CORT levels were measured immediately after exogenous CORT administration (Karten et al., 1999; Bush et al., 2003) or after exposure to stress (McKitterick et al., 1995). However, the CORT treated animals that were sacrificed at t=24 hr had a plasma corticosterone concentration which was significantly lower than the t=0 hr group. This may be attributed to suppression of the HPA axis (van Gemert et al., 2006) and is also consistent with the reduction in adrenal gland weight measured in the same animals (discussed in chapter 5).

It should be noted that whilst plasma corticosterone concentration in the CORT treated animals is in agreement with the available literature, the vehicle treated animals in the present study showed higher plasma CORT concentration than expected at both time points. This would imply that there may have been an acute release of corticosterone at around the time of sacrifice in vehicle treated rats although every attempt was made to reduce distress to animals. For example, the animals were sacrificed in a separate room to the one in which the remaining animals were housed and the duration between transport and sacrifice was minimised. However, a survey of the literature reveals that the mean value reported in this study (~100 ng/mL for the vehicle treated group) does not represent stress levels of corticosterone, which are reported to be 300-400 ng/mL in young adult male rats (Lister Hooded; body weight, 200-250g). In fact, the normal variation across the diurnal rhythm is between 37-65 ng/mL at the trough and 118-141 ng/mL at the peak in the same animals (Pinnock and Herbert, 2001). Although the vehicle treated rats showed a higher plasma corticosterone concentration than expected at the time of sacrifice (diurnal trough), it is still considerably lower than after exposure to stress, and in line with what is seen at the diurnal peak rather than after stress. In addition, it is important to consider that corticosterone is present in plasma bound to corticosterone binding globulin (CBG) and only about 10% is 'free' and bioavailable (Qian et al., 2011). After an acute stress, where plasma corticosterone concentration is increased, there is a 20-30 minute delay in the rise of free corticosterone in the brain (hippocampus and hypothalamus). This delay has been postulated to be a protective response and is related to an increase in the release of CBG from the liver rather than a delay due to transport across the BBB (Qian et al., 2011). Considering this in the

context of the data presented here, it is not expected that the short time between transport of animals and sacrifice (~3-4 minutes) would result in a sufficient increase in brain free corticosterone concentration to have impacted on the variables measured here. However, the possibility that this may have occurred cannot be completely excluded.

6.5.2 Oxytocin receptor binding

The regions selected for analysis of oxytocin receptor binding were selected as a mixture of limbic and extralimbic brain regions and also regions with overlapping vasopressin 1a and 5-HT_{1A} receptor distribution. Receptor autoradiography studies presented here revealed a high binding of [^{125}I]OVTA in regions consistent with the known receptor distribution of oxytocin receptors in the rat brain i.e. amygdala and septal nuclei, with lower levels of binding observed in cortical areas (Tribollet et al., 1988). In the present study, exogenous CORT administration resulted in a significant and marked increase in [^{125}I]OVTA binding in the septal nuclei and raphé, with a concomitant decrease in the hypothalamus. In addition, there was a trend towards an increase in binding in the amygdala after CORT treatment. Taken together these changes suggest that chronic exposure to corticosterone influences oxytocin receptor binding in a region-dependent manner.

The lateral septal nuclei are involved in regulating sexual behaviour, social recognition, learning and memory processes (Bielsky et al., 2005; Veenema et al., 2010; Curly et al., 2012). The septal nuclei primarily receives projections from the entorhinal cortex and hippocampus, therefore irregularities exist in mood disorders (Nolte, The Human Brain, 5th edn). The increase in oxytocin

receptor binding here is similar to that previously observed after dexamethasone treatment, where it was suggested that glucocorticoid modulation of oxytocin receptors may mediate the sexual dysfunction seen in depression (Patchev et al., 1993). In addition, the oxytocin receptor in the septal nuclei is implicated in regulating social and affective behaviours which are improved after oxytocin administration in rodents (Curly et al., 2012) and humans (Kosfeld et al., 2005).

As oxytocin is released in the septal nuclei in response to glucocorticoids, and has an inhibitory effect on the HPA axis thus terminating the stress response via attenuation of ACTH. This effect can be abolished by an oxytocin antagonist (Neumann et al., 2000; Neumann et al., 2002), and so our results indicate that upregulation of the oxytocin receptors is a compensatory event to initiate HPA re-activity.

In addition, here I report a significant increase in [^{125}I]OVTA binding in the raphé after chronic CORT treatment. The presence of oxytocin receptors in this region implicates links with serotonergic regulation and therefore disorders which involve the serotonin system i.e. mood disorders. Approximately 50% of the serotonergic neurons co-express oxytocin receptors and it is thought that they influence the release of 5-HT (Yoshida et al., 2009). It has been suggested that the release of oxytocin in the raphé could be part of a compensatory response to increase serotonin release in depression (Scantamburlo et al., 2007). Moreover, antidepressant treatments such as SSRIs have shown to cause a release of oxytocin in the brain, which may contribute to their efficacy (Uvnäs-Moberg et al., 1999). Although speculative, it is possible that glucocorticoids induce an upregulation of oxytocin receptors in the raphé which may induce such serotonergic changes.

There was an increase in [^{125}I]OVTA binding in the subiculum of the ventral hippocampus. This is consistent with Liberzon and Young (1997). The subiculum is one of the major sites of synaptic plasticity in the hippocampal formation. It connects the hippocampus to cortical regions and is associated with the inhibitory input to the HPA axis (O'Mara, 2005). Although, in the present study, the increase in [^{125}I]OVTA binding did not reach statistical significance, it is likely that this may be due to the small sample size that remained after sections of an inferior quality were removed. The subiculum is rich in MR and GR, therefore the tendency towards an increase in oxytocin receptor binding after exposure to corticosterone is of biological significance. It suggests that oxytocin may be involved in the inhibition of the HPA axis, possibly via interaction between the glucocorticoid receptors and the oxytocin receptors in the subiculum (Herman et al., 1992; Liberzon and Young, 1997).

Also in the present study, there was a tendency towards an increase in [^{125}I]OVTA binding in the amygdala. The amygdala is part of the limbic brain system and is considered to be the emotional processing centre. This region receives afferents carrying a huge amount of sensory data from several locations and projects to the cerebral cortex and hypothalamus (Nolte, The Human Brain, 5th edn). A high density of oxytocin receptors is found in the CeA subnuclei (Tribollet et al., 1988) and oxytocinergic neurones project to the hypothalamus suggesting a mechanism by which oxytocin may regulate the HPA axis (Stoop, 2012). In addition, this is a key brain region involved in the regulation of many processes such as fear and anxiety (Koo et al., 2004), memory consolidation and retrieval processes (de Oliviera et al., 2007) and also in stress coping mechanisms (Ebner et al., 2005). All these processes are affected in depression and so the changes in

oxytocin receptor binding observed in this study may be relevant for stress induced mood disorders. The tendency towards an increase in oxytocin receptor binding presented here is consistent with a previously reported increase after dexamethasone treatment (Patchev et al., 1993). Interestingly, oxytocin is released in the amygdala after exposure to forced swim stress, an effect that was attributed to passive stress-coping strategies. However, this was reversed after administration of an oxytocin antagonist suggesting a role for the oxytocin receptor in mediating these effects (Ebner et al., 2005). Taken together, this implies that glucocorticoids could potentially influence behaviour by modulating the oxytocin receptor in the amygdala, although this is speculative as behavioural responses were not investigated in this study.

Importantly, a bidirectional pathway between prefrontal cortex and amygdala has been described, through this the prefrontal cortex is able to exert an inhibitory influence on the amygdala (Miller et al., 2005). A neural mechanism has been postulated whereby the prosocial hormone oxytocin is responsible for governing prosocial behaviours by reducing feelings of fear in the amygdala (Debiec, 2007). It has been shown that oxytocin is released in response to stress and this results in an attenuation of ACTH release enforcing HPA re-activity (Neumann et al., 2000). Taken together with our results, this would suggest that the upregulation of oxytocin receptors in the amygdala may be a compensatory response to feelings of fear and anxiety and this could be mediated in part by the prefrontal cortex.

Furthermore, the central amygdaloid nucleus is where the majority of CRH pathways originate and there are also bidirectional pathways between CRH neurones from the amygdala to the hypothalamus and raphé. In addition some of

the amygdala CRH neurones also connect with serotonergic neurones (Gray, 1993). Therefore the amygdala CRH neurones are involved in the regulation of the HPA axis. Taken together with the results from this study, it is feasible that oxytocin receptors in the amygdala are part of the mechanism by which oxytocin exerts an inhibitory effect on the HPA axis in response to glucocorticoids.

In the present study, I also observed a decrease in the hypothalamic oxytocin receptor binding after CORT treatment, consistent with Patchev et al. (1993). The hypothalamus and septal nuclei are heavily interconnected suggesting they control similar functions (Stoop et al., 2012). The hypothalamus is also involved in the regulation of sexual behaviour and therefore the results presented here suggest that the sexual dysfunction seen in depression may be in part mediated via glucocorticoid modulation of the oxytocin receptor as also seen in the study of Patchev et al. (1999). More importantly, the decrease in oxytocin binding is significant considering that oxytocin inhibits the HPA axis. As mentioned above, the amygdala relays information to the hypothalamus and thus the observed reduction in binding at the oxytocin receptor in the hypothalamus, in response to glucocorticoids, may be part of the mechanism by which glucocorticoids influence HPA axis activity.

6.5.3 Oxytocin concentrations

The significant CORT-dependent decrease in oxytocin receptor binding in the hypothalamus demonstrated here was accompanied by a significant increase in oxytocin concentration in the region. This finding is important considering the tonic inhibitory role of oxytocin on the HPA axis (Neumann et al., 2000). The data presented here suggest that exposure to glucocorticoids leads

to an increase in oxytocin release in the hypothalamus, which in turn induces the known agonist stimulated internalisation of the oxytocin receptor, thus reducing the number of receptors available for binding. This may be part of the mechanism, by which oxytocin inhibits the HPA axis and is relevant to the anxiolytic function of oxytocin and in stress coping mechanisms (Neumann, 2000). However, it is accepted that this is speculative as receptor internalisation was not measured in this study.

The hypothalamus was the only brain region sampled to show a change in oxytocin content after CORT treatment. The fact that in the present study there was no effect of chronic glucocorticoid exposure on oxytocin content in the septal nuclei amygdala or raphé would suggest that glucocorticoids may be able to upregulate oxytocin receptors in these regions irrespective of peptide concentration. This was a somewhat surprising result considering the literature evidence that shows stress causes an increase in oxytocin release in the amygdala (Ebner et al., 2005) and septal nuclei (reviewed in Slattery and Neumann, 2010) as well as the hypothalamus (Wotjak et al., 1996). In addition, the present study did not show any significant changes in plasma oxytocin concentration after CORT treatment, although a ~40.5% increase was observed. It is possible that the plasma changes in oxytocin concentration may reach significance with a larger sample size.

6.5.4 Vasopressin V1a receptor binding

The regions selected for analysis of vasopressin 1a receptor binding were selected as a mixture of limbic and extralimbic brain regions and also regions with overlapping oxytocin and 5-HT_{1A} receptor distribution. Autoradiography

studies presented here revealed a high binding of [^{125}I]AVP in brain regions consistent with the known distribution of the vasopressin 1a receptor i.e. hypothalamus, septal nuclei, amygdala and thalamus, with lower levels of binding in the cortical areas (Tribollet et al., 1988). In the present study, exogenous CORT administration resulted in a significant and marked increase in [^{125}I]AVP binding in the LSD subregion of the septal nuclei and the PODG subregion of the dorsal hippocampus. In contrast, binding of [^{125}I]AVP was significantly decreased in the hypothalamus, suggesting that exposure to chronic corticosterone affects vasopressin 1a receptor binding in a region-specific manner. Although it is known that glucocorticoids regulate the expression of the V1a receptor, until now there has been only limited evidence on the effects of glucocorticoids on the V1a receptor across the rat brain (Watters et al., 1996).

The present study reports an increase in [^{125}I]AVP binding in the septal nuclei implying that glucocorticoids are able to modulate the vasopressin 1a receptor in this region. This is in agreement with the previously reported increase in V1aR in the septal nuclei after dexamethasone treatment (Watters et al., 1996). The septal nucleus is densely innervated by vasopressinergic neurones originating from the amygdala and expresses a high density of vasopressin 1a receptors (Stoop, 2012). In this region, the V1aR regulate processes such as the generation of emotions and also those associated with learning and memory (Ebner et al., 2002). The activation of the V1aR in the septal nuclei has been shown to be anxiogenic (Bielsky et al., 2005). Whereas, administration of a V1a receptor antagonist into the lateral septum has produced anxiolytic behavioural changes (Liebsch et al., 1996). Taken together, this suggests that glucocorticoids may be able to regulate behaviours associated with anxiety through modulation

of the vasopressin 1a receptor in the septal nuclei, although this remains speculative as behaviour was not investigated in this study.

This is the first report of the direct effects of chronic exposure to glucocorticoids on binding at the vasopressin 1a receptors in the dorsal hippocampus or raphé. The dorsal hippocampus, in particular the dentate gyrus complex, is involved in memory retrieval and learning processes (Matus-Amat et al., 2004). Vasopressin 1a receptor antagonism impairs memory retrieval, whereas agonists enhance memory retrieval (reviewed in Caldwell et al., 2008). Although speculative, chronic exposure to corticosterone may influence memory retrieval processes through an increase in expression of the vasopressin 1a receptor, however it is accepted that the dose of corticosterone used here was much higher than the physiological range.

Also, in the present study, a reduction in [^{125}I]AVP binding was observed in the hypothalamus. This is important when considered with the role of vasopressin as an activator of the HPA axis. Both vasopressin and CRH are secretagogues for adrenocorticotrophic hormone (ACTH), which in turn results in the release of glucocorticoids (Scott and Dinan, 1998). Approximately 50% of CRH neurones also express vasopressin and this proportion is greatly increased after exposure to a stressor, during which, vasopressin is the main regulator of the HPA axis response (Scott and Dinan, 1998; Aguilera et al., 2007). There is some evidence to show that the ensuing HPA axis re-activity mediated via glucocorticoids may inhibit vasopressin-induced ACTH release (Raff, 1987). Also, in the hypothalamus, glucocorticoids inhibit vasopressin expression at a transcriptional level (Erkut et al., 1998; Kovacs et al., 2000). Taken together, this

suggests that a reduction in vasopressin 1a receptors in the hypothalamus, as seen in the present study may be part of this mechanism.

6.5.5 Vasopressin concentrations

In the present study, administration with corticosterone for 21 days did not affect vasopressin concentrations in any brain region analysed nor in plasma. This would suggest that the upregulation of vasopressin 1a receptors in the septal nuclei, raphé and dorsal hippocampus may be independent of vasopressin concentration in these regions. Conversely, the reduction in binding observed in the hypothalamus also represents a downregulation of the V1aR. Interestingly, although not seen here, an increase in vasopressin content in the hypothalamus has been previously reported in depressed patients, which has correlated with the severity of disease (Merali et al., 2006). Thus, the reduction in binding may be a result of agonist induced internalisation of V1aR, although this is speculative as in the present study no change on vasopressin content was measured. However, it should be mentioned that the lack of effect on vasopressin content in both brain and plasma in the present study is a surprising result especially when extensive literature evidence suggests that exposure to stress (social defeat stress) results in a release of vasopressin as measured by microdialysis (Wotjak et al., 1996; reviewed in Scott and Dinan, 1998). Although technical and experimental differences are likely to provide an explanation for such discrepancies, the high variation seen in both the tissue and plasma analyses in this study may explain the lack of effect observed.

In conclusion, the data presented in this chapter suggest that chronic exposure to glucocorticoids modulates oxytocin and vasopressin 1a receptor

expression in extrahypothalamic regions in a way that is independent of endogenous peptide concentration. Glucocorticoid responsive elements have been identified on the genes of the oxytocin and vasopressin 1a receptor, providing a means by which corticosterone may modulate the expression of these receptors (Mohr and Schmitz, 1991; Liberzon and Young, 1997).

Chapter 7

Changes in binding at the cannabinoid receptor 1 after chronic exposure to glucocorticoids

There is increase evidence to suggest that the endocannabinoid system is involved in the regulation of the HPA axis either directly or through modulation of other neurotransmitter systems and therefore regulates responses to stress. Interestingly, patients treated with the anti-obesity drug Rimonabant (CB1 receptor antagonist) developed depression and anxiety-related disorders, possibly through increased activity of the HPA axis (Doyon et al., 2006). Thus endocannabinoid signalling via the CB1 receptor is important for the regulation of the HPA axis. This chapter describes a study investigating changes in CB1 receptor binding after chronic exposure to corticosterone in male rats and discusses these with regards to the serotonergic changes seen in chapter 5.

7.1 Introduction

Endocannabinoids are retrograde neuromodulators and are implicated in stress-related disorders (Roche and Finn, 2010). The endocannabinoid system has an inhibitory tone on the HPA axis, where disruption of endocannabinoid signalling leads to an increase in basal and stress-induced corticosterone release and an impairment of HPA axis reactivity (Di et al., 2003). Conversely, enhanced endocannabinoid signalling attenuates HPA axis activity (Di et al., 2003). Thus, in the normal brain, a high endocannabinoid concentration is found in the hypothalamus, which is thought to contribute to the basal inhibition of the HPA axis (Patel et al., 2004). However, exposure to chronic stress is associated with a reduction of endocannabinoid concentration in the prefrontal cortex, striata, amygdala, hippocampus and hypothalamus (Patel et al., 2004; Hill et al., 2008a) associated with an increase in serum corticosterone concentration and an increase in anxiety-like behaviour of rodents (Patel et al., 2004; reviewed in Roche and Finn, 2010). In contrast, a study by Di et al. (2005) found that endocannabinoid concentration was increased in the hypothalamus (PVN) after exposure to glucocorticoids and postulated that endocannabinoids are responsible for initiating the fast negative feedback mechanism of the HPA axis i.e. they mediate the nongenomic actions of glucocorticoids. Conventionally, it was thought that the glucocorticoid induced negative feedback mechanism is initiated by transcriptional control over the mediators of the HPA axis, however, it is now understood that a rapid action of glucocorticoids also exists that does not involve transcription and is therefore sometimes called ‘nongenomic mechanism’ or ‘fast feedback mechanism’ (Tasker et al., 2006). An elegant study by Di et al., 2003 postulates that glucocorticoids act on an as yet unknown

membrane bound G-protein coupled receptor on the PVN neurones. This results in an increase in endocannabinoid release which acts on the CB1 receptors on the glutamate neurones, inhibiting the release of glutamate and thus inhibiting the activity of the PVN and hormone secretion (see figure 7.1). This postulates a pivotal role for the CB1 receptor in the regulation of the HPA axis.

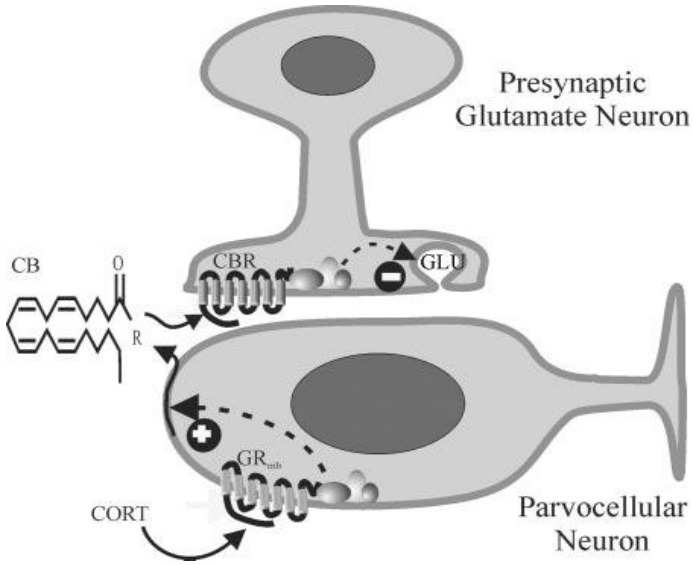


Figure 7.1 Mechanism of nongenomic regulation of the HPA axis in the PVN (Di et al., 2003).

7.1.1 The CB1 receptor and the stress response

The CB1 receptor is localised in limbic brain regions that are involved in the stress response and thus implicated in its regulation (Herkenham et al., 1991). CB1 receptors are also present in the adrenal gland where they can regulate the release of glucocorticoids peripherally (Galiegue et al., 1995). The most compelling evidence for an involvement of the CB1 receptor in the regulation of the HPA axis is that pretreatment with the CB1 antagonist SR141716A inhibits Δ^9- tetrahydrocannabinol (THC, a CB1 receptor agonist)

induced ACTH release (Manzanares et al., 2009). In addition, the generation of CB1 receptor knockout mice has further enhanced the understanding of the relationship between the CB1 receptor and regulation of the HPA axis. Indeed CB1 receptor knockout mice display characteristic anxiogenic and depressive behaviours which cannot be ameliorated with antidepressants (Uriguen et al., 2004). In addition, CB1 knockout mice have a flattening of the circadian rhythm whereby corticosterone concentration remain elevated throughout. This suggests that the inhibitory tone on HPA axis activity is diminished with the loss of CB1 receptor signalling. Therefore the CB1 receptor is required for basal modulation of the HPA axis (Cota et al., 2007).

Further to this, the administration of several CB1 receptor antagonists to rats, leads to an increase in the secretion of corticosterone (Weidenfield et al., 1994; Wenger et al., 1997). Also, corticosterone secretion is increased in the amygdala after *in situ* antagonism of the CB1 receptor, whereas administration of HU-210, a CB1 agonist decreases corticosterone release (Hill et al., 2009).

More importantly in the context of this study, the CB1 receptor is under negative regulation by glucocorticoids. Chronic unpredictable stress, for 21 days downregulates CB1 receptors in the hippocampus (Hill et al., 2005). However, in contrast, adrenalectomy upregulates CB1 receptor mRNA in the striata which is reversed upon glucocorticoid replacement (Mailleux and Vanderhaeghen, 1993). As mentioned above, this suggests that the fast glucocorticoid negative feedback mechanism of the HPA axis is modulated by the CB1 receptor (Di et al., 2003).

In this chapter, I outline a study designed to investigate the effect of exposure to corticosterone via addition to drinking water for 21 days on CB1

receptor binding. The radioligand [^{3}H]CP 55,940 was used which has an affinity between 0.5-5nM and is extensively used to study the CB1 receptor (Pertwee, 2005). It was not possible to measure the concentrations of endocannabinoids in discrete brain regions according to the design in chapters 5 and 6, due to their very short half-life and methodology; this would require analysis via mass spectrometry, a technique which fell outside of the scope of this study.

7.2 Aims and Hypothesis

7.2.1 Aims

The endocannabinoid system has a tonic inhibitory control over the HPA axis (Cota et al., 2007) an effect which is mediated via the CB1 receptor. In fact, CB1 receptor knockout mice as well as administration of a CB1 receptor antagonist resulted in an increase in corticosterone secretion (Cota et al., 2007; Hill et al., 2005). Stressful paradigms have been shown to reduce endocannabinoid concentration in the brain (Patel et al., 2004; Hill et al., 2008a). Although exposure to glucocorticoids has also been shown to result in an increase after glucocorticoid administration (Di et al., 2005). However, the influence of glucocorticoids on CB1 receptor binding in extralimbic regions has been seldom studied. In this thesis, I aim to investigate the effects of glucocorticoids on CB1 receptor binding in regions that are involved in responses to stress. The endocannabinoid and in particular the CB1 receptor is involved in maintaining the GABA-Glutamate balance and is also involved in regulation of the serotonergic system via the raphé (Rossi et al., 2008; Haj-Dahmane and Shen, 2009). Due to the pivotal role of endocannabinoid regulation over two of the major systems involved in depression, an

investigation into the influence of glucocorticoids on binding to the CB1 receptor is warranted.

7.2.2 Hypothesis

It is hypothesised that chronic exposure to high concentrations of glucocorticoid for 21 days will result in changes to CB1 receptor binding in brain regions that are involved in the regulation of responses to stress. The direction of changes in receptor binding is expected to reflect changes in the concentration of endocannabinoids, according to the previously published literature. More specifically, it is hypothesised that chronic exposure to glucocorticoids will result in an decrease in CB1 receptor binding in amygdala and hippocampus amongst other brain regions associated with the regulation of responses to stress.

7.3 Materials and Methods

All materials used for this study are given in chapter 4 Also, in chapter 4, are methodological details on *in-vivo* husbandry, dosing on animals, collection of post mortem samples, tissue sectioning and autoradiography methodology.

7.4 Results

Changes in CB1 receptor binding were quantified after chronic exposure to CORT. The quantitative data showing [^{3}H]CP 55,940 specific binding to CB1 receptors expressed as pmol/mg tissue are shown in table 7.1. A two way ANOVA was used to test for effects of treatment and also for region. Significant

differences were found for both treatment ($F(1,248) = 3.88$, $P = 0.0499$) and region. ($F(18,248) = 82.88$, $P = <0.0001$). The interaction was not significant. Further analysis between treatment groups in each region revealed significant differences in three regions sampled (two-tailed Student's t-test with Bonferroni at $P = 0.05$). These are shown in figure 7.2. A significant difference in regional uptake was seen in , the striata and the raphé. The significant 34.7% decrease in the striata after CORT treatment held significance after post hoc correction. Likewise, the raphé showed a 46.5% decrease in CB1 receptor binding retaining significance after Bonferroni correction. In addition, there was a tendency towards a decrease in [^{3}H]CP 55,940 binding in the PVN ($P<0.1$). Figure 7.3 shows representative autoradiograms of brain sections from vehicle and CORT treated rats labeled with [^{3}H]CP 55,940. Alongside, are images from brain sections showing non-specific binding i.e. those which were incubated in the presence of 10 µM Rimonabant.

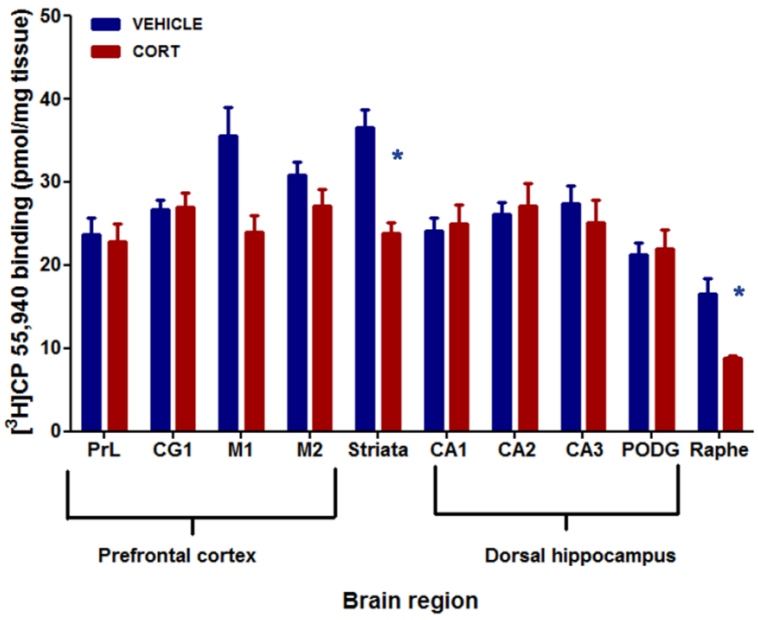


Figure 7.2 Effects of chronic corticosterone on [^{3}H]CP 55,940 binding in rat brain. Animals were treated with either vehicle (0.8% ethanol) or corticosterone (400 µg/mL) for 21 days. Animals were sacrificed after a 24 hour hormone free period. Data from regions showing a differences after corticosterone treatment are shown and expressed as mean ± SEM (n=8 per treatment group, except hypothalamus and subiculum, where n=5 per treatment group due to the tissue becoming damaged during the experiment which meant that the regions could not be sampled accurately). Abbreviations for regions are the same as in figure 4.5. Statistical analysis was carried out using 2-way ANOVA for treatment effects and regional effects followed by Bonferroni post hoc test for multiple comparisons, was applied with a significance level of 0.05. A statistically significant effect after correction is denoted by *.

Table 7.1 Regional [^{3}H]CP 55,940 binding in rat brain after chronic exposure to corticosterone.

Brain region	Vehicle treated group (pmol/mg tissue)	CORT treated group (pmol/mg tissue)	% change	P value
Hypothalamus				
VMDH	10.69 ± 4.38	7.96 ± 2.15	-25.6	0.2456
PVN	9.10 ± 1.51	7.01 ± 1.83	-23.0	0.0841
Prefrontal cortex				
PrL	23.70 ± 5.84	22.85 ± 6.18	-3.6	0.7824
CgL	26.64 ± 3.54	26.98 ± 4.99	+1.2	0.8798
M2	30.83 ± 4.61	27.20 ± 5.54	-11.7	0.1760
M1	35.53 ± 9.73	24.02 ± 5.45	-32.4	0.0112
Nucleus Accumbens	15.05 ± 5.51	18.09 ± 4.05	+20.2	0.2291
Shell	15.05 ± 5.51	15.42 ± 3.76	+2.5	0.8780
Core				
Striata	36.53 ± 6.19	23.85 ± 3.50 *	-34.7	0.0002
Amygdala	14.18 ± 1.86	14.24 ± 3.27	+0.4	0.9644
Dorsal hippocampus				
CA1 rad	24.16 ± 6.54	25.01 ± 6.26	+3.5	0.7596
CA2 rad	26.10 ± 3.94	27.14 ± 7.86	+3.9	0.7438
CA3 rad	27.43 ± 5.98	25.13 ± 7.69	-8.4	0.5152
PODG	21.32 ± 3.78	21.93 ± 6.80	+2.9	0.8281
Ventral hippocampus				
CA1 rad	30.74 ± 9.16	32.80 ± 6.12	+6.7	0.6053
Subiculum	29.52 ± 3.39	30.78 ± 3.70	+4.3	0.5896
Substantia Nigra	147.5 ± 55.76	130.0 ± 33.54	-11.9	0.4580
Raphé	16.55 ± 5.08	8.86 ± 0.80 *	-46.5	0.0008
Entorhinal cortex	18.44 ± 5.67	15.43 ± 3.84	-16.3	0.2338

Table 7.1 Animals were treated in the same way as described in figure 7.4. Data are expressed as mean ± SD (n=8 per treatment group, except hypothalamus and subiculum, where n=5 per treatment group due to the tissue becoming damaged during the experiment which meant that the regions could not be sampled accurately). Abbreviations used for regions are the same as in figure 4.5. Statistical analysis was carried out using 2-way ANOVA for treatment effects and regional effects followed by Bonferroni post hoc test for multiple comparisons, with a significance level of 0.05. A statistically significant effect after correction is denoted by *.

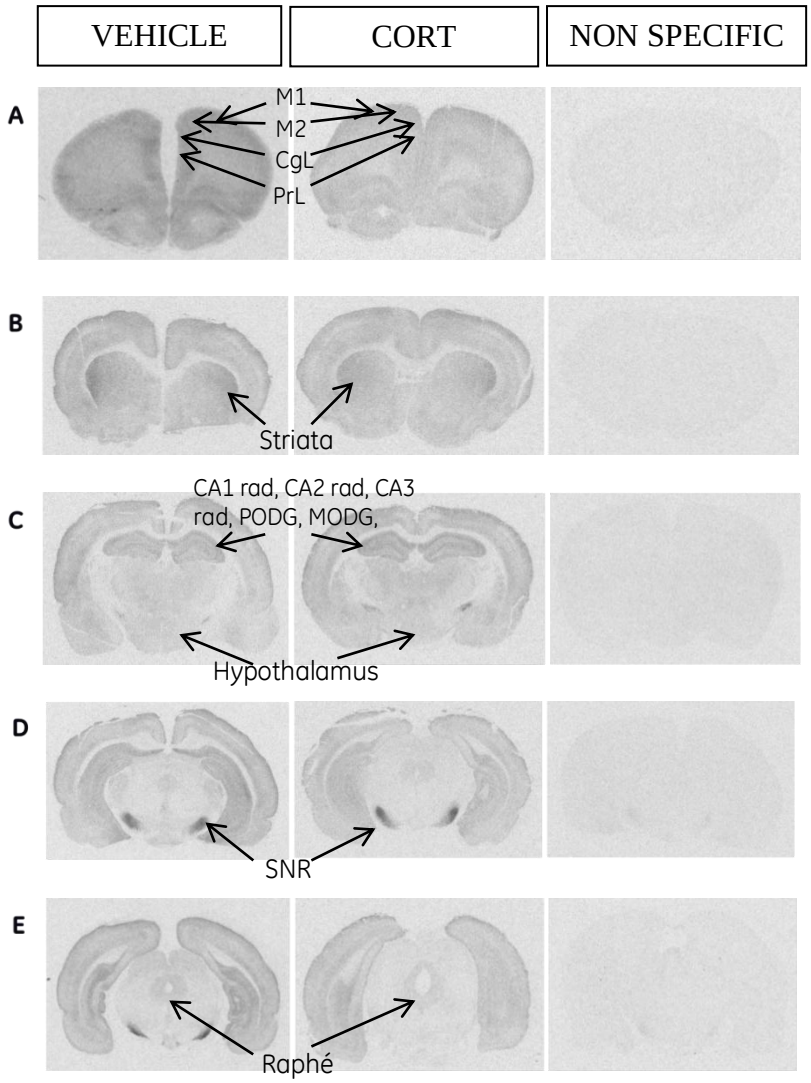


Figure 7.3 Representative [^{3}H]CP 55,940 autoradiography images from rat brain. Measured after chronic exposure to corticosterone as described in figure 7.4. Images are from individual rats representing A = prefrontal cortex (PrL, CgL, M1, M2), B = Striata, Nucleus accumbens (shell and core), C = dorsal hippocampus (CA1 rad, CA2 rad, CA3 rad, MODG, PODG, amygdala, VMDH), D = CA1 rad of ventral hippocampus, subiculum, substantia nigra (SNR), E = raphé. Further details of ROI location and sampling can be found in chapter 4.

7.5 Discussion

The present study demonstrates that long-term exposure to exogenous corticosterone induces changes in endocannabinoid receptor binding. Here, I present data quantifying the effects of exogenous corticosterone on CB1 receptor binding throughout the brain, in both the limbic and cortical regions. Corticosterone treatment affected CB1 receptors in brain regions known to be involved in responses to stress. Receptor autoradiography studies presented here reveal a high binding of [^{3}H]CP 55,940 in striatum, substantia nigra, CA3 and dentate gyrus subregion of dorsal hippocampus, which is consistent with the known receptor distribution of CB1 receptors in rat brain (Herkenham et al., 1991). In the present study, exogenous CORT administration resulted in a significant decrease in [^{3}H]CP 55,940 binding in the striatum and raphé.

The striatum is an important region for regulating emotional and cognitive functions which are affected by stress. In addition, the striatum is particularly abundant in CB1 receptors (Herkenham et al., 1991) and these have been reported to be involved in mediating the anxiolytic effects of endocannabinoids (Rossi et al., 2008). Here, I report a reduction in striatal CB1 receptor binding after chronic exposure to glucocorticoids. Since endocannabinoid concentration in this region was not achievable within the scope of this thesis, the interpretation of how the CB1 receptor is modulated by glucocorticoids is speculative. However, Hill et al. (2008a) showed a significant reduction in anandamide concentration (measured after extraction from dissected tissue) in the striata after 21 days of chronic unpredictable stress, and also a decrease in CB1 receptor density. Although it is accepted that in the present study chronic exposure to corticosterone may not result in an increase in

endocannabinoid concentration in the same way, the decrease in [^{3}H]CP 55,940 binding here is consistent with the above mentioned finding of Hill et al. (2008a). In the striatum, the CB1 receptors are located on presynaptic neurones which influence both GABA and glutamate release (Rossi et al., 2010). Exposure of mice to chronic social defeat stress or corticosterone (10mg/kg for 3 days) results in desensitisation of those CB1 receptors which regulate GABA release, but no change is seen in those CB1 receptors which regulate glutamate release (Rossi et al., 2008). This suggests that glucocorticoids disrupt the GABA-glutamate balance, which is implicated in depression, by altering endocannabinoid signalling in the striata (Rossi et al., 2008). Thus, the results from the present study and that of Hill et al. (2008) suggest that corticosterone induced desensitisation of CB1 receptors may result in reduced expression of the CB1 receptors in the striatum.

Also, in the present study, there was a reduction in [^{3}H]CP 55,940 binding in the raphé. The effect of glucocorticoids on the raphé CB1 receptor has not been reported previously. The location of CB1 receptors in the raphé implies that these are able to influence central serotonergic activity. Indeed, activation of the CB1 receptors in the raphé inhibits serotonin release, modulates the firing rate of raphé serotonergic neurons (Nakazi et al., 2000; Tzavara et al., 2003) and reduces serotonin content in projection regions such as the prefrontal cortex and hippocampus (Egashira et al., 2002). This has shown to be via retrograde inhibition of glutamate release (Haj-Dahmane and Shen, 2009). Furthermore, pharmacological blockade of the CB1 receptor enhances basal extracellular 5-HT in the prefrontal cortex (Haj-Dahmane, 2011). The results from the present study indicate that chronic exposure to glucocorticoids impair the

endocannabinoid receptor activity in the raphé, which can putatively affect the regulation of the serotonergic system. Although speculative, when considered with the lack of change in 5-HT, 5-HIAA and 5-HT turnover seen in this investigation (outlined in chapter 5), it suggests that the decrease in CB1 receptor binding reported in this study could be the reason that no change in 5-HT content was observed. That is, it may represent a mechanism by which 5-HT content is maintained in projection regions after exposure to corticosterone for 21 days.

Importantly, there was a tendency towards a decrease in [^{3}H]CP 55,940 binding in the PVN after corticosterone treatment. It is possible that this may reach significance with a larger sample size, bearing in mind that in this study, some of these sections were damaged during the autoradiography procedure. Therefore the final analysis here is from n=5 in both treatment groups. However, this finding is potentially relevant as it suggests that glucocorticoids may be able to modulate the expression of the CB1 receptor in the PVN. Di et al. (2003) have shown that corticosterone exerts an inhibitory effect on the HPA axis via endocannabinoid release resulting in reduced binding at the CB1 receptor possibly due to internalisation of the receptor. They also show that this effect is abolished by a CB1 receptor antagonist. Although speculative, it is possible that a downregulation of the CB1 receptor may be part of the mechanism of HPA axis dysfunction. However, it is accepted that a significant finding is needed to add strength to this.

Against the hypothesis, the study presented in this chapter did not show a significant change in CB1 receptor binding in the hippocampus. There are a few reports demonstrating a decrease in CB1 receptors in the hippocampus after

stress (Hill et al., 2008a, b; Hill and McEwen, 2010b). The hippocampus is highly susceptible to stress induced structural and neurochemical changes (Magarinos et al., 1998) and hence the development of depression (McEwen, 2003). There is a high density of CB1 receptors in the hippocampus which are located on GABAergic interneurons (Herkenham et al., 1991). The lack of glucocorticoid induced change in [^{3}H]CP 55,940 binding in this study compared to that observed in Hill et al. (2008a and b) may be explained by different treatment conditions and differences in the strain of rat used. Studies by Hill et al. are in Long Evans or Sprague Dawley rats, whereas here, Wistar rats were used. Strain differences in the responses to stress and its mediators are well established (Gómez et al., 1996; Wu and Wang, 2010). In addition there were differences in the autoradiography methodology between the two studies. Although the protocols were similar, the studies of Hill et al. have determined non-specific binding with unlabelled CP 55,940 whereas in the current study non-specific binding was determined using a saturating dose of the selective CB1 antagonist, rimonabant (SR141716A). It is expected that at the high dose used here it will also block the CB2 receptors.

It should be taken into consideration that CP 55,940 binds with similar affinity to both CB1 and CB2 receptors, and the fact that both these receptors are present in the rat hippocampus which could impede the results presented here (Gong et al., 2006; Brusco et al., 2008). However, it is thought that the CB2 receptor density is much lower than that of the CB1 receptor (Gong et al., 2006; Onaivi et al., 2006) as measured by a 100 fold lower expression of CB2 receptor mRNA. Although that study investigated the relative expression in mice, there is evidence to suggest the expression of CB2 receptor transcripts in the rat brain is

100 fold lower than that in the rat spleen (Onaivi et al., 2006). Therefore it is assumed that the specific binding of [^{3}H]CP 55,940 seen in this study represents binding to the CB1 receptor as the relative contribution by CB2 receptors is minimal due to the much lower expression levels.

The findings presented in this chapter suggest that CB1 receptor binding is reduced by glucocorticoid overload in a region specific manner and due to the interactions between the endocannabinoid and serotonergic systems, this may be responsible for the serotonergic effects reported in chapter 5.

Chapter 8

General Discussion

Stress related mood disorders are common. It is estimated that approximately 1 in 6 people are affected by depression at any given time in the UK (The Office for National Statistics, 2000). Not only is depression widespread, but it is an extremely costly disorder (Sobocki et al., 2006), partly due to difficulties in accurate diagnosis and treatment. It is clear that a greater understanding of the underlying neurobiological dysfunction in depression is needed as well as more accurate diagnosis (Pajer et al., 2012). A common feature of depression is a hyperactivity of the HPA axis resulting from elevated glucocorticoid concentrations (Anacker et al., 2011) which has been shown to lead to neurological deficits associated with depression.

In this thesis, I attempted to understand the impact of an increase in circulating glucocorticoid concentration on four neurotransmitter systems known

to regulate the HPA axis. In particular the effects of chronically elevated glucocorticoid concentrations on the neuropeptide and endocannabinoid systems is investigated and their influence on the serotonergic system. This is important as irregularities in each of these are associated with depressive symptoms and a lot of effort has been placed on determining the exact nature of these abnormalities with a view to improving patient diagnosis and treatment (Slattery and Neumann, 2010; Caldwell et al., 2008; Carrasco et al., 2003; Hill and McEwen, 2010). To that end, molecular imaging techniques such as PET and SPECT may be applied to further investigate the receptor systems in conditions of elevated glucocorticoid, however, the question remains as to what exactly needs to be imaged. It was envisaged that the neurotransmitter systems studied here may provide a biologically relevant target that could be imaged to provide information which could help in the management of depressed patients that also present with HPA axis abnormalities.

Attempts to develop radioligands for imaging the glucocorticoid receptor have so far failed, and this approach is particularly challenging because of the low expression of the glucocorticoid receptors in the human brain (30-40 fmol/mg protein) (reviewed in Steiniger et al., 2008). Therefore, in order to image these receptors, radioligands with an extremely high affinity (<1 nM) are required. This is problematic as it means that a very high specific activity is also required since competition of the binding sites by endogenous ligand needs to be considered (reviewed in Steiniger et al., 2008). In addition, a high specific activity radiopreparation is also required to avoid significant receptor occupancy by unlabelled compound that is co-administered (Hume at al., 1998; reviewed in Steiniger et al., 2008). Furthermore, the compounds investigated to date have

shown poor metabolic stability and/or a high degree of non specific binding hampering further their utility. Due to the above mentioned problems with imaging the glucocorticoid receptor directly, in this thesis, changes in the 5-HT_{1A}, oxytocin, vasopressin 1a and CB1 receptors were investigated after chronic exposure to corticosterone. Furthermore, as these neurotransmitter systems are known to be interconnected, the influence of glucocorticoid induced changes to the neuropeptidergic and endocannabinoid systems on the serotonergic system are discussed to determine if these interactions are relevant for the understanding of stress induced depression. I have attempted to describe the changes that I measured in this study in figure 8.1.

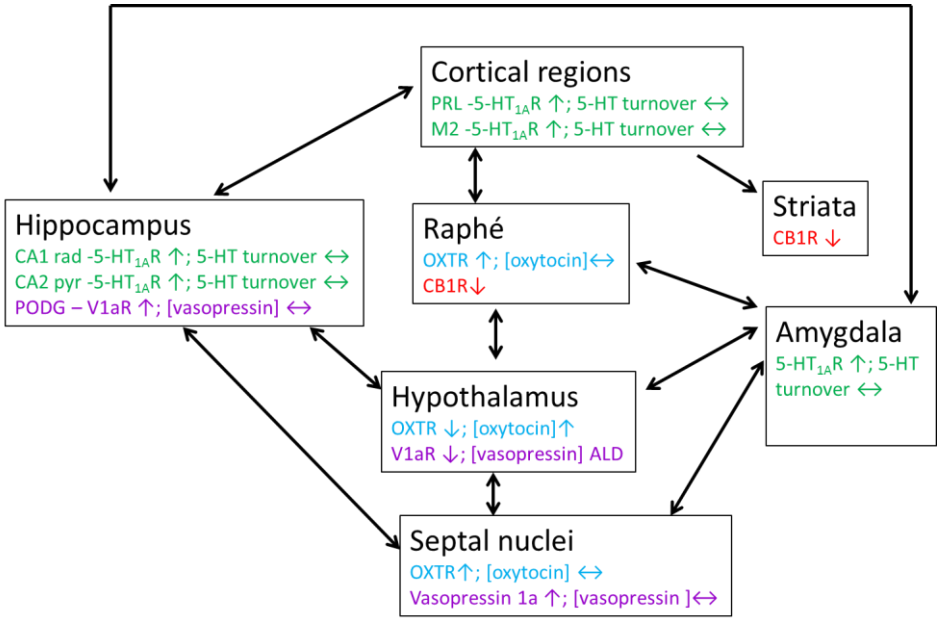


Figure 8.1 A simple schematic diagram showing significant changes measured in receptor binding and neurotransmitter concentration for each of the systems studied in this study. The relevant regions and their interactions are shown. Abbreviations: 5-$HT1_AR$ = 5-$HT1_A$ receptor, OXTR = oxytocin receptor, V1aR = vasopressin 1a receptor; CB1R = CB1 receptor. Arrows indicate the direction of change observed after chronic exposure to corticosterone. These changes may be relevant for the regulation of the HPA axis. Methodological details for the experiments performed are given in chapter 4.

In summary, I measured an upregulation of the 5-HT_{1A} receptor in the PRL and M2 subregion of the prefrontal cortex, amygdala, CA1 rad and CA2 pyr subregions of the dorsal hippocampus. There was no associated change in 5-HT turnover in any region. I also measured an upregulation of the oxytocin receptor in the septal nuclei and raphé, however, there was no change in the oxytocin concentration measured here. Interestingly, there was a decrease in oxytocin receptor binding in the hypothalamus which was associated with a decrease in oxytocin content in the same region. In addition, the vasopressin 1a receptor was upregulated in the PODG subregion of the dorsal hippocampus and septal nuclei without a concomitant increase in vasopressin content. However the vasopressin 1a receptor binding was decreased in the hypothalamus. Furthermore, the CB1 receptor was decreased in the striata and raphé.

For the serotonergic system, I reported an increase in 5-HT_{1A} receptor binding in postsynaptic regions; PRL and M2 subregion of the prefrontal cortex, amygdala, CA1 rad and CA2 pyr subregions of the dorsal hippocampus. This increase in [^{3}H]WAY 100635 binding, measured using in vitro autoradiography was not associated with a change in serotonin concentration suggesting the possibility of an upregulation of the 5-HT_{1A} receptor in response to elevated glucocorticoids. Although this is contradictory to the majority of the published literature, where a decrease in 5-HT_{1A} receptor binding has been reported, there is one major difference between this study and those reported. That is, here an antagonist radioligand is used, whereas other studies used the agonist [^{3}H]8-OHDPAT. Thus, because [^{3}H]WAY 100635 binds to both the low and high affinity states of the 5-HT_{1A} receptor, the total receptor population is considered and not just a subpopulation. The results presented in this thesis suggest that

chronic exposure to glucocorticoid for 21 days is sufficient to cause an upregulation of the total number of 5-HT_{1A} receptors which may represent an attempt to 'prime' the serotonergic system i.e. to ensure there are enough receptors present to easily convert to the high affinity state when needed (Strange, 2000).

The upregulation of 5-HT_{1A} receptors in the PRL and M2 subregion of the prefrontal cortex may represent prefrontal control over raphé serotonergic neurones as a bidirectional pathway is known to exist between these regions (Martin-Ruiz et al., 2001). Whilst the upregulation of 5-HT_{1A} receptors in the CA1 rad and CA2 pyr subregions of the dorsal hippocampus could represent a neuroprotective mechanism against a stress induced increase in glutamate during stress.

At present, there is a successful antagonist radioligand for imaging the 5-HT_{1A} receptor; [^{11}C]WAY 100635 (Pike et al., 1996; Bantick et al., 2004; Saigal et al., 2006; Tipre et al., 2006). However, the carbon-11 label limits its clinical utility. An F-18 radiolabelled antagonist has been developed; [^{18}F]MPPF but its fast clearance and higher nonspecific binding component when compared to [^{11}C]WAY 100635 hinders its use beyond a basic research tool (Wooten et al., 2011). Interestingly, recent PET studies using [^{11}C]WAY100635 have shown that those patients which responded to the SSRI, escitalopram had a 33% higher baseline 5-HT_{1A} receptor baseline than those patients that did not respond to the therapy (Miller et al., 2013). Furthermore, depressed patients with a higher 5-HT_{1A} receptor binding potential showed remission after 3 months of psychopharmacotherapy (Lan et al., 2013). However, these studies did not look at HPA axis function in the subjects, so it remains possible that a glucocorticoid

induced increase in 5-HT_{1A} receptor binding as measured here could predict therapy response and hence could also aid in therapy selection for depressed patients. There has been one PET study to date looking at the effect of glucocorticoids on 5-HT_{1A} receptor binding, which failed to find a relationship, although it should be borne in mind that the subjects in the study were not considered to be clinically depressed (Montgomery et al., 2001).

Investigation of the neuropeptide systems showed that chronic corticosterone exposure altered the expression of oxytocin receptors in a region specific manner. A decrease in oxytocin receptor binding observed in the hypothalamus, was also accompanied by an increase in oxytocin concentration. It is possible that this decrease in binding is due to downregulation of the oxytocin receptor, in response to the increase in oxytocin content of the hypothalamus after treatment with corticosterone. In other regions however, oxytocin receptor binding was increased (septal nuclei and raphe), without an associated change in peptide concentration suggesting that corticosterone may induce an upregulation in the expression of oxytocin receptors in these regions. This is an important finding when considered along with the fact that the oxytocin receptor is coexpressed on serotonergic neurones in the raphé and that this receptor is involved in the regulation of the serotonergic system (Yoshida et al., 2009). Moreover, SSRI administration is known to induce the release of oxytocin (Uvnäs-Moberg et al., 1999). Therefore, the upregulation measured in the current study after chronic glucocorticoid exposure may represent a compensatory mechanism to maintain forebrain serotonin concentrations and may explain the lack of effect on the concentrations of 5-HT, 5-HIAA and 5-HT turnover. Furthermore, the upregulation of oxytocin receptors in the septal

nuclei, is also significant as oxytocinergic neurones project from this region to the hypothalamus where they exert an inhibitory influence on the HPA axis (Neumann et al., 2000; 2002), thus this pathway may represent a mechanism by which HPA axis reactivity is initiated.

With respect to the value of imaging the oxytocin receptor in depression, the significantly large increases in oxytocin receptor binding measured here in the raphé (+100%) and septal nuclei (+87.5%) after corticosterone treatment suggest that PET imaging of this receptor in response to glucocorticoids could be amenable. Attempts have already been made to generate F-18 and C-11 radioligands for the oxytocin receptor. Smith et al. (2012) report the synthesis of four non-peptide small molecule antagonists for the oxytocin receptor. Unfortunately, all four compounds failed to show utility for imaging central oxytocin receptors *in vivo* due to low brain uptake and so further development was halted. However, the study by Smith et al. (2012) does provide a good chemistry starting point for the synthesis of related compounds with improved *in vivo* characteristics.

As with oxytocin receptor binding, specific binding to the vasopressin 1a receptor measured after elevated circulating corticosterone concentration was also affected in a region-specific manner. A decrease in [^{125}I]AVP binding was observed in the hypothalamus, but specific binding was increased in the septal nuclei and PODG subregion of the dorsal hippocampus. These changes were not associated with a change in vasopressin content in the same regions, suggesting that the vasopressin 1a receptor may also be upregulated or downregulated in response to glucocorticoids. These findings are relevant as vasopressin is a known activator of the HPA axis and therefore the downregulation of

vasopressin 1a receptors seen in this region here suggests that this is part of the mechanism by which HPA axis re-activity is initiated. Furthermore, raphé serotonergic neurones project to the hypothalamus and synapse with vasopressinergic neurones (Ferris and Deville, 1999). Bearing in mind that I did not measure a change in 5-HT_{1A} receptor number or 5-HT turnover in the raphé suggests that serotonergic function is maintained in the region and thus may influence HPA axis reactivity via influencing the vasopressin 1a receptor in the hypothalamus. In addition, activation of the vasopressin 1a receptor in the septal nuclei has an anxiogenic effect (Bielsky et al., 2005) and thus chronic glucocorticoid exposure may result in an increase in anxiogenic behaviours via an upregulation of vasopressin 1a receptors in the septal nuclei. However, the septal nuclei also projects to the hypothalamus and vasopressin neurones may be responsible for regulating the HPA axis (Stoop, 2012). Importantly, this is the first time that it has been shown that glucocorticoids affect the vasopressin 1a receptor response in the dorsal hippocampus. The increase in [^{125}I]AVP binding in the PODG subregion of the dorsal hippocampus suggests that a glucocorticoid induced upregulation of the vasopressin 1a receptor may be involved in the learning and memory impairments seen during chronic stress (Caldwell et al., 2008).

As with the oxytocin receptor, the degree of increase in binding at the vasopressin 1a receptor demonstrated here in the septal nuclei (+30.0%) and PODG of the dorsal hippocampus (+78.6%) could be detected by PET, however, as far as I am aware, there are no published data on the evaluation of radioligands for the vasopressin 1a receptor. There is one confounding issue with imaging this target in stress induced depression which is related to the resolution

of the currently available PET scanners especially when used without associated MRI coregistration. Subregional analysis of the dorsal hippocampus, for example, to measure the vasopressin 1a receptor effect seen here in the PODG may need some methodology work up as the effect is likely be lost if the whole dorsal hippocampus volume of interest is sampled.

Finally, chronic corticosterone treatment was also shown to influence endocannabinoid signalling through the CB1 receptor. Binding of [^{3}H]CP 55,940 was reduced in the striata and raphé suggesting that exposure to glucocorticoids may modulate CB1 receptor expression, and thus contribute to the serotonergic abnormalities seen in depression. The decreased binding to CB1 receptors in the striata may contribute to the GABA-Glutamate imbalance which is seen in depression. Furthermore, the reduced CB1 receptor binding seen in the raphé implies a link with the regulation of the serotonergic system. Indeed, activation of the CB1 receptors in the raphé inhibits serotonin release, modulates the firing rate of raphé serotonergic neurons (Nakazi et al., 2000; Tzavara et al., 2003) and reduces serotonin content in projection regions such as the prefrontal cortex and hippocampus (Egashira et al., 2002). This has shown to be via retrograde inhibition of glutamate release (Haj-Dahmane and Shen, 2009). This is the first time that the effects of glucocorticoids on raphé CB1 receptors has been reported and when considered alongside the lack of change in 5-HT, 5-HIAA and 5-HT turnover, suggests that the decrease in CB1 receptor binding reported in this study could be the reason that no change in 5-HT content was observed. That is, it may represent a mechanism by which 5-HT content is maintained in projection regions after exposure to corticosterone for 21 days.

Due to the fact that the decreases in CB1 receptor binding observed in the striata (-34.7%) and also in the raphé (-46.5%) are relatively large it would be interesting to see if these changes could be detected by PET imaging. The development of radiotracers for the CB1 receptor has progressed further than the neuropeptide systems. Initial attempts were hampered by a high degree of lipophilicity exhibited by the radioligands, which is problematic considering the brain has a ~60% lipid composition, thus reducing the brain uptake and specific signal of these radioligands (Yasuno et al., 2008). However, since then, two carbon-11 labelled radioligands, [^{11}C]OMAR and [^{11}C]MEPPEP (Yasuno et al., 2008) showed more promise. [^{11}C]OMAR has only been tested in preclinical studies, and is yet to be evaluated in humans (Herance et al., 2011), whereas [^{11}C]MEPPEP displays slow kinetics which is a major drawback for use in humans (Fan et al., 2009). More recently, [^{18}F]MK-9470, a CB1 receptor ligand was evaluated in rats, but development was halted due to the presence of radiolabelled metabolites, which cross the BBB and contaminate the signal (Casteels et al., 2012).

9.1 Future work

The results from the present study suggest that *in vivo* non-invasive dosing of animals with corticosterone via drinking water can be used for further preclinical studies investigating the influence of glucocorticoids on a number of neurotransmitter systems including the serotonergic, neuropeptidergic and endocannabinoid systems. It would be interesting to understand the impact that this treatment had on the glucocorticoid receptor, however, at present this is

challenging due to the lack of suitable radioligands. Although a measurement of glucocorticoid receptor mRNA may provide some insight.

In addition, it would be interesting to compare the results obtained here with those derived from stress paradigms i.e. HPA axis activation via application of a psycho-physical stressor such as restraint stress. Such a paradigm would offer a complete HPA axis effect, with CRF and ACTH rather than from glucocorticoid alone. In addition, as behavioural studies were not conducted here, it would be interesting and relevant to assess the behavioural effects of either paradigm in parallel to the neurobiological ones. Also, gender effects could be studied preclinically as these are known to exist for the oxytocin and vasopressin systems and are relevant as women are more susceptible to depression than males. Furthermore, it would be interesting to assess whether the reported changes in receptor expression are affected by SSRI treatment, particularly as these drugs have been shown to act partly via the oxytocinergic system (Uvnäs-Moberg et al., 1999). This could be investigated preclinically by dosing rats with corticosterone as described here, followed by 31 days of fluoxetine treatment (Leventopoulos et al., 2009).

The data presented herein suggest that the 5-HT_{1A}, extrahypothalamic oxytocin and extrahypothalamic vasopressin 1a receptors respond to elevated levels of glucocorticoids and hence could be of relevance in disorders such as stress induced depression. Taking into consideration the multifaceted approach to understand depression and other related mood disorders, the ability to detect abnormalities in neurotransmitter systems other than the serotonergic system is attractive. The changes measured here in the oxytocinergic, vasopressinergic and endocannabinoid systems is important as these systems are known to interact

and influence serotonergic regulation (Ferris and Deville, 1999; Uvnäs Moberg et al., 1999; Haj-Dahmane, 2011) and therefore may be relevant in stress induced mood disorders.

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Appendix B

Feasibility organ weights

Parameter	
Table Analyzed	SC adrenal (mg)/100 g body weight (g)
Column A	VEHICLE
vs	vs
Column B	CORT
Unpaired t test	
P value	< 0.0001
P value summary	****
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=7.378 df=10
How big is the difference?	
Mean ± SEM of column A	10.38 ± 0.6292 N=6
Mean ± SEM of column B	3.681 ± 0.6544 N=6
Difference between means	6.699 ± 0.9079
95% confidence interval	4.676 to 8.721
R square	0.8448

Parameter	
Table Analyzed	SC thymus (mg)/100 g body weight (g)
Column A	VEHICLE
vs	vs
Column B	CORT
Unpaired t test	
P value	< 0.0001
P value summary	****
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=7.825 df=10
How big is the difference?	
Mean ± SEM of column A	172.9 ± 6.431 N=6
Mean ± SEM of column B	85.71 ± 9.091 N=6
Difference between means	87.14 ± 11.14
95% confidence interval	62.33 to 112.0
R square	0.8596

Parameter	
Table Analyzed	drink adrenal (mg)/100 g body weight (g)
Column A	VEHICLE
vs	vs
Column B	CORT
Unpaired t test	
P value	< 0.0001
P value summary	****
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=8.806 df=10
How big is the difference?	
Mean ± SEM of column A	19.27 ± 1.374 N=6
Mean ± SEM of column B	6.865 ± 0.3094 N=6
Difference between means	12.40 ± 1.409
95% confidence interval	9.265 to 15.54
R square	0.8858

Parameter	
Table Analyzed	drink thymus (mg)/100 g body weight (g)
Column A	VEHICLE
Vs	vs
Column B	CORT
Unpaired t test	
P value	0.0007
P value summary	***
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=4.839 df=10
How big is the difference?	
Mean ± SEM of column A	178.7 ± 7.659 N=6
Mean ± SEM of column B	91.29 ± 16.36 N=6
Difference between means	87.40 ± 18.06
95% confidence interval	47.16 to 127.7
R square	0.7007

Feasibility weight gain

Parameter				
Table Analyzed	sc injection grp			
Two-way ANOVA				
Source of Variation	% of total variation	P value		
Interaction	21.04	< 0.0001		
Treatment	52.20	< 0.0001		
duration	17.79	< 0.0001		
Source of Variation	P value summary	Significant?		
Interaction	****	Yes		
Treatment	****	Yes		
Duration	****	Yes		
Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	20	40753	2038	24.64
Treatment	1	101120	101120	1223
duration	20	34465	1723	20.84
Residual	210	17364	82.68	

Parameter				
Table Analyzed	drink grp			
Two-way ANOVA				
Source of Variation	% of total variation	P value		
Interaction	16.58	< 0.0001		
Treatment	49.35	< 0.0001		
Day	24.28	< 0.0001		
Source of Variation	P value summary	Significant?		
Interaction	****	Yes		
Treatment	****	Yes		
Duration	****	Yes		
Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	20	40903	2045	17.80
Treatment	1	121748	121748	1059
Day	20	59907	2995	26.06
Residual	210	24134	114.9	

Water consumed

Parameter				
Table Analyzed	water consumed (ml/rat)			
Two-way ANOVA				
Source of Variation	% of total variation	P value		
Interaction	3.82	0.8548		
treatment	9.82	0.0010		
duration	37.33	< 0.0001		
Source of Variation	P value summary	Significant?		
Interaction	ns	No		
treatment	***	Yes		
duration	****	Yes		
Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	9	218.5	24.27	0.5195
treatment	1	561.8	561.8	12.02
duration	9	2134	237.2	5.076
Residual	60	2804	46.73	

Parameter				
Table Analyzed	ml/kgwater consumed			
Two-way ANOVA				
Source of Variation	% of total variation	P value		
Interaction	7.63	< 0.0001		
treatment	18.34	< 0.0001		
duration	25.50	< 0.0001		
Source of Variation	P value summary	Significant?		
Interaction	****	Yes		
treatment	****	Yes		
duration	****	Yes		
Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	9	43412	4824	5.242
treatment	1	104344	104344	113.4
duration	9	145047	16116	17.51
Residual	300	276079	920.3	

Parameter	
Table Analyzed	ttest (ml/rat) day3
Column A	VEHICLE
vs	vs
Column B	CORT
Unpaired t test	
P value	0.5911
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=0.5674 df=6
How big is the difference?	
Mean ± SEM of column A	66.50 ± 2.723 N=4
Mean ± SEM of column B	64.00 ± 3.464 N=4
Difference between means	2.500 ± 4.406
95% confidence interval	-8.283 to 13.28
R square	0.05092

Parameter	
Table Analyzed	ttest (ml/rat) day5
Column A	VEHICLE
Vs	vs
Column B	CORT
Unpaired t test	
P value	0.1954
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=1.457 df=6
How big is the difference?	
Mean ± SEM of column A	63.25 ± 3.521 N=4
Mean ± SEM of column B	57.25 ± 2.136 N=4
Difference between means	6.000 ± 4.118
95% confidence interval	-4.077 to 16.08
R square	0.2613

Parameter	
Table Analyzed	ttest (ml/rat) day7
Column A	VEHICLE
vs	vs
Column B	CORT
Unpaired t test	
P value	0.6291
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=0.5087 df=6
How big is the difference?	
Mean ± SEM of column A	64.00 ± 3.651 N=4
Mean ± SEM of column B	61.75 ± 2.496 N=4
Difference between means	2.250 ± 4.423
95% confidence interval	-8.573 to 13.07
R square	0.04135

Parameter	
Table Analyzed	ttest (ml/rat) day9
Column A	VEHICLE
vs	vs
Column B	CORT
Unpaired t test	
P value	0.0825
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=2.082 df=6
How big is the difference?	
Mean ± SEM of column A	66.25 ± 4.571 N=4
Mean ± SEM of column B	56.00 ± 1.826 N=4
Difference between means	10.25 ± 4.922
95% confidence interval	-1.795 to 22.29
R square	0.4195

Parameter	
Table Analyzed	ttest (ml/rat) day11
Column A	VEHICLE
vs	vs
Column B	CORT
Unpaired t test	
P value	0.4173
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=0.8709 df=6
How big is the difference?	
Mean ± SEM of column A	63.50 ± 4.173 N=4
Mean ± SEM of column B	59.25 ± 2.529 N=4
Difference between means	4.250 ± 4.880
95% confidence interval	-7.691 to 16.19
R square	0.1122

Parameter	
Table Analyzed	ttest (ml/rat) day13
Column A	VEHICLE
vs	vs
Column B	CORT
Unpaired t test	
P value	0.4383
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=0.8299 df=6
How big is the difference?	
Mean ± SEM of column A	60.25 ± 1.887 N=4
Mean ± SEM of column B	57.50 ± 2.723 N=4
Difference between means	2.750 ± 3.313
95% confidence interval	-5.358 to 10.86
R square	0.1030

Parameter	
Table Analyzed	ttest (ml/rat) day15
Column A	VEHICLE
vs	vs
Column B	CORT
Unpaired t test	
P value	0.2492
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=1.276 df=6
How big is the difference?	
Mean ± SEM of column A	62.25 ± 2.136 N=4
Mean ± SEM of column B	56.50 ± 3.969 N=4
Difference between means	5.750 ± 4.507
95% confidence interval	-5.278 to 16.78
R square	0.2134

Parameter	
Table Analyzed	ttest (ml/rat) day17
Column A	VEHICLE
vs	vs
Column B	CORT
Unpaired t test	
P value	0.0007
P value summary	***
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=6.419 df=6
How big is the difference?	
Mean ± SEM of column A	64.75 ± 1.702 N=4
Mean ± SEM of column B	52.25 ± 0.9465 N=4
Difference between means	12.50 ± 1.947
95% confidence interval	7.735 to 17.26
R square	0.8729

Parameter	
Table Analyzed	ttest (ml/rat) day19
Column A	VEHICLE
vs	vs
Column B	CORT
Unpaired t test	
P value	0.6841
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=0.4273 df=6
How big is the difference?	
Mean ± SEM of column A	75.50 ± 7.089 N=4
Mean ± SEM of column B	72.00 ± 4.103 N=4
Difference between means	3.500 ± 8.190
95% confidence interval	-16.54 to 23.54
R square	0.02954

Parameter	
Table Analyzed	ttest (ml/rat) day21
Column A	VEHICLE
vs	vs
Column B	CORT
Unpaired t test	
P value	0.5581
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=0.6200 df=6
How big is the difference?	
Mean ± SEM of column A	73.75 ± 3.637 N=4
Mean ± SEM of column B	70.50 ± 3.775 N=4
Difference between means	3.250 ± 5.242
95% confidence interval	-9.577 to 16.08
R square	0.06021

Parameter	
Table Analyzed	ttest (ml/kg) day3
Column A	VEHICLE
vs	vs
Column B	CORT
Unpaired t test	
P value	0.1642
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=1.426 df=30
How big is the difference?	
Mean ± SEM of column A	287.9 ± 5.569 N=16
Mean ± SEM of column B	300.7 ± 7.106 N=16
Difference between means	-12.88 ± 9.029
95% confidence interval	-31.31 to 5.561
R square	0.06349

Parameter	
Table Analyzed	ttest (ml/kg) day3
Column A	VEHICLE
vs	vs
Column B	CORT
Unpaired t test	
P value	0.1642
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=1.426 df=30
How big is the difference?	
Mean ± SEM of column A	287.9 ± 5.569 N=16
Mean ± SEM of column B	300.7 ± 7.106 N=16
Difference between means	-12.88 ± 9.029
95% confidence interval	-31.31 to 5.561
R square	0.06349

Parameter	
Table Analyzed	ttest (ml/kg) day7
Column A	VEHICLE
vs	vs
Column B	CORT
Unpaired t test	
P value	0.0005
P value summary	***
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=3.912 df=30
How big is the difference?	
Mean ± SEM of column A	251.4 ± 8.031 N=16
Mean ± SEM of column B	288.9 ± 5.226 N=16
Difference between means	-37.48 ± 9.582
95% confidence interval	-57.05 to -17.92
R square	0.3378

Parameter	
Table Analyzed	ttest (ml/kg) day9
Column A	VEHICLE
vs	vs
Column B	CORT
Unpaired t test	
P value	0.5160
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=0.6573 df=30
How big is the difference?	
Mean ± SEM of column A	253.4 ± 9.836 N=16
Mean ± SEM of column B	261.0 ± 6.017 N=16
Difference between means	-7.578 ± 11.53
95% confidence interval	-31.12 to 15.97
R square	0.01420

Parameter	
Table Analyzed	ttest (ml/rat) day11
Column A	VEHICLE
vs	vs
Column B	CORT
Unpaired t test	
P value	0.0021
P value summary	**
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=3.367 df=30
How big is the difference?	
Mean ± SEM of column A	236.1 ± 9.294 N=16
Mean ± SEM of column B	274.0 ± 6.308 N=16
Difference between means	-37.82 ± 11.23
95% confidence interval	-60.76 to -14.88
R square	0.2742

Parameter	
Table Analyzed	ttest (ml/kg) day13
Column A	VEHICLE
vs	vs
Column B	CORT
Unpaired t test	
P value	< 0.0001
P value summary	****
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=5.100 df=30
How big is the difference?	
Mean ± SEM of column A	215.3 ± 5.151 N=16
Mean ± SEM of column B	262.1 ± 7.597 N=16
Difference between means	-46.81 ± 9.178
95% confidence interval	-65.56 to -28.07
R square	0.4644

Parameter	
Table Analyzed	ttest (ml/kg) day15
Column A	VEHICLE
vs	vs
Column B	CORT
Unpaired t test	
P value	0.0009
P value summary	***
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=3.687 df=30
How big is the difference?	
Mean ± SEM of column A	215.8 ± 5.071 N=16
Mean ± SEM of column B	255.9 ± 9.606 N=16
Difference between means	-40.05 ± 10.86
95% confidence interval	-62.23 to -17.87
R square	0.3118

Parameter	
Table Analyzed	ttest (ml/kg) day17
Column A	VEHICLE
vs	vs
Column B	CORT
Unpaired t test	
P value	0.0271
P value summary	*
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=2.323 df=30
How big is the difference?	
Mean ± SEM of column A	220.2 ± 5.214 N=16
Mean ± SEM of column B	235.6 ± 4.091 N=16
Difference between means	-15.40 ± 6.628
95% confidence interval	-28.93 to -1.863
R square	0.1525

Parameter	
Table Analyzed	ttest (ml/kg) day19
Column A	VEHICLE
vs	vs
Column B	CORT
Unpaired t test	
P value	0.0001
P value summary	***
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=4.352 df=30
How big is the difference?	
Mean ± SEM of column A	251.8 ± 12.85 N=16
Mean ± SEM of column B	324.5 ± 10.70 N=16
Difference between means	-72.77 ± 16.72
95% confidence interval	-106.9 to -38.63
R square	0.3870

Parameter	
Table Analyzed	ttest (ml/kg) day21
Column A	VEHICLE
vs	vs
Column B	CORT
Unpaired t test	
P value	< 0.0001
P value summary	****
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=6.896 df=30
How big is the difference?	
Mean ± SEM of column A	240.5 ± 6.351 N=16
Mean ± SEM of column B	317.3 ± 9.149 N=16
Difference between means	-76.81 ± 11.14
95% confidence interval	-99.55 to -54.06
R square	0.6132

Body weight

Parameter				
Table Analyzed	body weight (g)			
Two-way ANOVA				
Source of Variation	% of total variation	P value		
Interaction	10.85	< 0.0001		
treatment	51.17	< 0.0001		
duration	18.91	< 0.0001		
Source of Variation	P value summary	Significant?		
Interaction	****	Yes		
treatment	****	Yes		
duration	****	Yes		
Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	10	49986	4999	18.77
treatment	1	235825	235825	885.4
duration	10	87138	8714	32.71
Residual	330	87899	266.4	

Parameter	
Table Analyzed	day 1
Column A	VEHICLE
vs	vs
Column B	CORT
Unpaired t test	
P value	0.0647
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=1.918 df=30
How big is the difference?	
Mean ± SEM of column A	220.8 ± 0.9895 N=16
Mean ± SEM of column B	213.8 ± 3.479 N=16
Difference between means	6.938 ± 3.617
95% confidence interval	-0.4479 to 14.32
R square	0.1092
Are variances significantly different?	Yes

Parameter	
Table Analyzed	day 3
Column A	VEHICLE
vs	vs
Column B	CORT
Unpaired t test	
P value	0.0001
P value summary	***
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=4.393 df=30
How big is the difference?	
Mean ± SEM of column A	231.2 ± 1.646 N=16
Mean ± SEM of column B	213.3 ± 3.721 N=16
Difference between means	17.88 ± 4.069
95% confidence interval	9.566 to 26.18
R square	0.3914

Parameter	
Table Analyzed	day 5
Column A	VEHICLE
vs	vs
Column B	CORT
Unpaired t test	
P value	< 0.0001
P value summary	****
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=7.640 df=30
How big is the difference?	
Mean ± SEM of column A	247.8 ± 3.024 N=16
Mean ± SEM of column B	212.7 ± 3.452 N=16
Difference between means	35.06 ± 4.589
95% confidence interval	25.69 to 44.43
R square	0.6605

Parameter	
Table Analyzed	day 7
Column A	VEHICLE
vs	vs
Column B	CORT
Unpaired t test	
P value	< 0.0001
P value summary	****
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=8.139 df=30
How big is the difference?	
Mean ± SEM of column A	255.7 ± 3.608 N=16
Mean ± SEM of column B	214.2 ± 3.603 N=16
Difference between means	41.50 ± 5.099
95% confidence interval	31.09 to 51.91
R square	0.6883

Parameter	
Table Analyzed	day 9
Column A	VEHICLE
vs	vs
Column B	CORT
Unpaired t test	
P value	< 0.0001
P value summary	****
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=8.618 df=30
How big is the difference?	
Mean ± SEM of column A	263.1 ± 4.247 N=16
Mean ± SEM of column B	215.5 ± 3.525 N=16
Difference between means	47.56 ± 5.519
95% confidence interval	36.29 to 58.83
R square	0.7123

Parameter	
Table Analyzed	day 11
Column A	VEHICLE
vs	vs
Column B	CORT
Unpaired t test	
P value	< 0.0001
P value summary	****
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=9.091 df=30
How big is the difference?	
Mean ± SEM of column A	270.9 ± 4.706 N=16
Mean ± SEM of column B	217.0 ± 3.614 N=16
Difference between means	53.94 ± 5.933
95% confidence interval	41.82 to 66.05
R square	0.7337

Parameter	
Table Analyzed	day 13
Column A	VEHICLE
vs	vs
Column B	CORT
Unpaired t test	
P value	< 0.0001
P value summary	****
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=9.321 df=30
How big is the difference?	
Mean ± SEM of column A	281.4 ± 5.261 N=16
Mean ± SEM of column B	220.5 ± 3.870 N=16
Difference between means	60.88 ± 6.531
95% confidence interval	47.54 to 74.21
R square	0.7433

Parameter	
Table Analyzed	day 15
Column A	VEHICLE
vs	vs
Column B	CORT
Unpaired t test	
P value	< 0.0001
P value summary	****
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=11.12 df=30
How big is the difference?	
Mean ± SEM of column A	289.8 ± 5.022 N=16
Mean ± SEM of column B	221.9 ± 3.456 N=16
Difference between means	67.81 ± 6.096
95% confidence interval	55.36 to 80.26
R square	0.8049

Parameter	
Table Analyzed	day 17
Column A	VEHICLE
vs	vs
Column B	CORT
Unpaired t test	
P value	< 0.0001
P value summary	****
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=10.98 df=30
How big is the difference?	
Mean ± SEM of column A	295.9 ± 5.679 N=16
Mean ± SEM of column B	222.6 ± 3.497 N=16
Difference between means	73.25 ± 6.669
95% confidence interval	59.63 to 86.87
R square	0.8008

Parameter	
Table Analyzed	day 19
Column A	VEHICLE
vs	vs
Column B	CORT
Unpaired t test	
P value	< 0.0001
P value summary	****
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=11.08 df=30
How big is the difference?	
Mean ± SEM of column A	302.7 ± 6.071 N=16
Mean ± SEM of column B	223.1 ± 3.840 N=16
Difference between means	79.56 ± 7.184
95% confidence interval	64.89 to 94.23
R square	0.8035

Parameter	
Table Analyzed	day 21
Column A	VEHICLE
vs	vs
Column B	CORT
Unpaired t test	
P value	< 0.0001
P value summary	****
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=12.28 df=30
How big is the difference?	
Mean ± SEM of column A	308.1 ± 5.966 N=16
Mean ± SEM of column B	223.0 ± 3.519 N=16
Difference between means	85.06 ± 6.927
95% confidence interval	70.92 to 99.21
R square	0.8341

Organ weights

Parameter	
Table Analyzed	adrenal (mg)/100 g body weight (g)
Column A	VEHICLE
vs	vs
Column B	CORT
Unpaired t test	
P value	0.0017
P value summary	**
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=3.444 df=30
How big is the difference?	
Mean ± SEM of column A	22.22 ± 0.8250 N=16
Mean ± SEM of column B	15.98 ± 1.613 N=16
Difference between means	6.239 ± 1.812
95% confidence interval	2.540 to 9.939
R square	0.2834

Parameter	
Table Analyzed	thymus (mg)/100 g body weight (g)
Column A	VEHICLE
vs	vs
Column B	CORT
Unpaired t test	
P value	< 0.0001
P value summary	****
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=4.560 df=30
How big is the difference?	
Mean ± SEM of column A	162.6 ± 7.413 N=16
Mean ± SEM of column B	110.8 ± 8.587 N=16
Difference between means	51.73 ± 11.34
95% confidence interval	28.57 to 74.90
R square	0.4094

Table Analyzed	adrenal in mgs
Column A	VEHICLE
vs	vs
Column B	CORT
Unpaired t test	
P value	< 0.0001
P value summary	****
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=7.686 df=30
How big is the difference?	
Mean ± SEM of column A	67.16 ± 2.617 N=16
Mean ± SEM of column B	34.69 ± 3.317 N=16
Difference between means	32.48 ± 4.225
95% confidence interval	23.85 to 41.10
R square	0.6632

Table Analyzed	Thymus in mgs
Column A	VEHICLE
vs	vs
Column B	CORT
Unpaired t test	
P value	< 0.0001
P value summary	****
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=8.124 df=30
How big is the difference?	
Mean ± SEM of column A	493.2 ± 25.59 N=16
Mean ± SEM of column B	242.1 ± 17.32 N=16
Difference between means	251.1 ± 30.90
95% confidence interval	188.0 to 314.2
R square	0.6875

5-HT$_{1A}$ receptor autoradiography

Table Analyzed	stats			
Two-way ANOVA				
Source of Variation	% of total variation	P value		
Interaction	5.85	0.0007		
treatment	7.04	< 0.0001		
region	55.74	< 0.0001		
Source of Variation	P value summary	Significant?		
Interaction	***	Yes		
treatment	****	Yes		
region	****	Yes		
Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	15	17418	1161	2.740
treatment	1	20976	20976	49.49
region	15	166002	11067	26.11
Residual	214	90706	423.9	

Parameter	
Table Analyzed	CA1 rad
Column A	veh
vs	vs
Column B	cort
Unpaired t test	
P value	0.0010
P value summary	**
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=4.117 df=14
How big is the difference?	
Mean ± SEM of column A	63.18 ± 2.275 N=8
Mean ± SEM of column B	85.14 ± 4.824 N=8
Difference between means	-21.96 ± 5.334
95% confidence interval	-33.40 to -10.52
R square	0.5476

Parameter	
Table Analyzed	CA2 rad
Column A	veh
vs	vs
Column B	cort
Unpaired t test	
P value	0.0038
P value summary	**
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=3.458 df=14
How big is the difference?	
Mean ± SEM of column A	61.94 ± 5.875 N=8
Mean ± SEM of column B	104.5 ± 10.82 N=8
Difference between means	-42.57 ± 12.31
95% confidence interval	-68.98 to -16.16
R square	0.4606

Parameter	
Table Analyzed	CA2 pyr
Column A	veh
vs	vs
Column B	cort
Unpaired t test	
P value	0.0017
P value summary	**
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=3.874 df=14
How big is the difference?	
Mean ± SEM of column A	45.35 ± 2.649 N=8
Mean ± SEM of column B	81.65 ± 8.986 N=8
Difference between means	-36.29 ± 9.369
95% confidence interval	-56.39 to -16.20
R square	0.5174

Parameter	
Table Analyzed	CA3 rad
Column A	veh
vs	vs
Column B	cort
Unpaired t test	
P value	0.0112
P value summary	*
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=2.920 df=14
How big is the difference?	
Mean ± SEM of column A	55.29 ± 4.781 N=8
Mean ± SEM of column B	111.2 ± 18.54 N=8
Difference between means	-55.91 ± 19.15
95% confidence interval	-96.98 to -14.83
R square	0.3785

Parameter	
Table Analyzed	MODG
Column A	veh
vs	vs
Column B	cort
Unpaired t test	
P value	0.6589
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=0.4510 df=14
How big is the difference?	
Mean ± SEM of column A	96.91 ± 8.687 N=8
Mean ± SEM of column B	101.3 ± 4.381 N=8
Difference between means	-4.388 ± 9.729
95% confidence interval	-25.26 to 16.48
R square	0.01432

Parameter	
Table Analyzed	Amygdala
Column A	veh
vs	vs
Column B	cort
Unpaired t test	
P value	0.0027
P value summary	**
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=3.627 df=14
How big is the difference?	
Mean ± SEM of column A	28.31 ± 2.493 N=8
Mean ± SEM of column B	51.28 ± 5.821 N=8
Difference between means	-22.97 ± 6.333
95% confidence interval	-36.55 to -9.385
R square	0.4844

Parameter	
Table Analyzed	pir3
Column A	veh
vs	vs
Column B	cort
Unpaired t test	
P value	0.0177
P value summary	*
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=2.686 df=14
How big is the difference?	
Mean ± SEM of column A	26.17 ± 2.444 N=8
Mean ± SEM of column B	54.85 ± 10.39 N=8
Difference between means	-28.68 ± 10.68
95% confidence interval	-51.58 to -5.776
R square	0.3401

Parameter	
Table Analyzed	PrL
Column A	veh
vs	vs
Column B	cort
Unpaired t test	
P value	0.0031
P value summary	**
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=3.573 df=14
How big is the difference?	
Mean ± SEM of column A	34.61 ± 1.196 N=8
Mean ± SEM of column B	60.51 ± 7.149 N=8
Difference between means	-25.90 ± 7.249
95% confidence interval	-41.45 to -10.35
R square	0.4770

Parameter	
Table Analyzed	CGL
Column A	veh
vs	vs
Column B	cort
Unpaired t test	
P value	0.0317
P value summary	*
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=2.386 df=14
How big is the difference?	
Mean ± SEM of column A	40.69 ± 5.290 N=8
Mean ± SEM of column B	57.32 ± 4.530 N=8
Difference between means	-16.62 ± 6.965
95% confidence interval	-31.56 to -1.681
R square	0.2892

Parameter	
Table Analyzed	M2
Column A	veh
vs	vs
Column B	cort
Unpaired t test	
P value	0.0006
P value summary	***
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=4.437 df=14
How big is the difference?	
Mean ± SEM of column A	37.50 ± 2.166 N=8
Mean ± SEM of column B	62.73 ± 5.258 N=8
Difference between means	-25.23 ± 5.687
95% confidence interval	-37.43 to -13.04
R square	0.5844

Parameter	
Table Analyzed	M1
Column A	veh
vs	vs
Column B	cort
Unpaired t test	
P value	0.0442
P value summary	*
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=2.210 df=14
How big is the difference?	
Mean ± SEM of column A	37.49 ± 3.257 N=8
Mean ± SEM of column B	54.54 ± 6.993 N=8
Difference between means	-17.05 ± 7.714
95% confidence interval	-33.60 to -0.5042
R square	0.2587

Parameter	
Table Analyzed	Raphe
Column A	veh
vs	vs
Column B	cort
Unpaired t test	
P value	0.4407
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=0.7935 df=14
How big is the difference?	
Mean ± SEM of column A	112.6 ± 6.890 N=8
Mean ± SEM of column B	103.7 ± 8.830 N=8
Difference between means	8.887 ± 11.20
95% confidence interval	-15.14 to 32.91
R square	0.04304

Parameter	
Table Analyzed	ent ctx
Column A	veh
vs	vs
Column B	cort
Unpaired t test	
P value	0.2012
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=1.341 df=14
How big is the difference?	
Mean ± SEM of column A	61.31 ± 5.232 N=8
Mean ± SEM of column B	71.68 ± 5.701 N=8
Difference between means	-10.38 ± 7.738
95% confidence interval	-26.98 to 6.220
R square	0.1139

Parameter	
Table Analyzed	Vhip CA1
Column A	veh
vs	vs
Column B	cort
Unpaired t test	
P value	0.9011
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=0.1266 df=14
How big is the difference?	
Mean ± SEM of column A	93.94 ± 11.81 N=8
Mean ± SEM of column B	96.25 ± 13.97 N=8
Difference between means	-2.315 ± 18.29
95% confidence interval	-41.55 to 36.92
R square	0.001143

Parameter	
Table Analyzed	subiculum
Column A	veh
vs	vs
Column B	cort
Unpaired t test	
P value	0.2928
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=1.117 df=9
How big is the difference?	
Mean ± SEM of column A	8.343 ± 1.128 N=5
Mean ± SEM of column B	12.51 ± 3.243 N=6
Difference between means	-4.164 ± 3.727
95% confidence interval	-12.60 to 4.267
R square	0.1218

Parameter	
Table Analyzed	hypothalamus
Column A	veh
vs	vs
Column B	cort
Unpaired t test	
P value	0.3076
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=1.082 df=9
How big is the difference?	
Mean ± SEM of column A	23.88 ± 4.734 N=5
Mean ± SEM of column B	17.32 ± 3.890 N=6
Difference between means	6.557 ± 6.063
95% confidence interval	-7.157 to 20.27
R square	0.1150

5HIAA concentration

Parameter				
Table Analyzed	5HIAA stats layout			
Two-way ANOVA				
Source of Variation	% of total variation	P value		
Interaction	0.23	0.9992		
treatment	0.37	0.3518		
region	54.07	< 0.0001		
Source of Variation	P value summary	Significant?		
Interaction	ns	No		
treatment	ns	No		
region	****	Yes		
Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	7	53.79	7.684	0.07851
treatment	1	85.59	85.59	0.8746
region	7	12599	1800	18.39
Residual	108	10570	97.87	

5-HT content

Parameter				
Table Analyzed	5HT stats layout			
Two-way ANOVA				
Source of Variation	% of total variation	P value		
Interaction	4.07	0.0166		
treatment	0.88	0.0507		
region	71.06	< 0.0001		
Source of Variation	P value summary	Significant?		
Interaction	*	Yes		
treatment	ns	No		
region	****	Yes		
Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	7	732.3	104.6	2.587
treatment	1	157.8	157.8	3.903
region	7	12781	1826	45.15
Residual	108	4367	40.43	

Serotonin turnover

Parameter				
Table Analyzed	turnover stats layout			
Two-way ANOVA				
Source of Variation	% of total variation	P value		
Interaction	6.25	0.1053		
treatment	0.08	0.6909		
region	38.56	< 0.0001		
Source of Variation	P value summary	Significant?		
Interaction	ns	No		
treatment	ns	No		
region	****	Yes		
Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	7	1.106	0.1580	1.749
treatment	1	0.01436	0.01436	0.1589
region	7	6.820	0.9743	10.78
Residual	108	9.761	0.09038	

Corticosterone concentration (RIA)

Parameter				
Table Analyzed	Corticosterone 2way ANOVA			
Two-way ANOVA				
Source of Variation	% of total variation	P value		
Interaction	26.17	< 0.0001		
Treatment	8.19	0.0051		
Timepoint	26.34	< 0.0001		
Source of Variation	P value summary	Significant?		
Interaction	****	Yes		
Treatment	**	Yes		
Timepoint	****	Yes		
Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	1	400423	400423	28.04
Treatment	1	125328	125328	8.775
Timepoint	1	403080	403080	28.22
Residual	41	585575	14282	

Parameter	
Table Analyzed	0hrs VEH vs Cort
Column A	Vehicle 0hrs
vs	vs
Column B	Corticosterone 0hrs
Unpaired t test	
P value	0.0083
P value summary	**
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=3.073 df=14
How big is the difference?	
Mean ± SEM of column A	109.6 ± 37.99 N=7
Mean ± SEM of column B	419.1 ± 83.20 N=9
Difference between means	-309.5 ± 100.7
95% confidence interval	-525.5 to -93.46
R square	0.4028

Parameter	
Table Analyzed	24hrsVEH vs Cort
Column A	Vehicle 24hrs
vs	vs
Column B	Corticosterone 24hrs
Unpaired t test	
P value	< 0.0001
P value summary	****
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=7.469 df=27
How big is the difference?	
Mean ± SEM of column A	109.0 ± 12.19 N=13
Mean ± SEM of column B	21.54 ± 3.748 N=16
Difference between means	87.42 ± 11.71
95% confidence interval	63.41 to 111.4
R square	0.6738

Parameter	
Table Analyzed	veh 0&24hrs
Column A	Vehicle 0hrs
vs	vs
Column B	veh 24 hrs
Unpaired t test	
P value	0.9838
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=0.02056 df=18
How big is the difference?	
Mean ± SEM of column A	109.6 ± 37.99 N=7
Mean ± SEM of column B	109.0 ± 12.19 N=13
Difference between means	0.6575 ± 31.98
95% confidence interval	-66.54 to 67.86
R square	2.347e-005

Parameter	
Table Analyzed	cort 0&24hrs
Column A	Corticosterone 0hrs
vs	vs
Column B	Corticosterone 24hrs
Unpaired t test	
P value	< 0.0001
P value summary	****
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=6.460 df=23
How big is the difference?	
Mean ± SEM of column A	419.1 ± 83.20 N=9
Mean ± SEM of column B	21.54 ± 3.748 N=16
Difference between means	397.5 ± 61.54
95% confidence interval	270.2 to 524.9
R square	0.6447

Oxytocin receptor autoradiography

Table Analyzed	veh vs cort all regions			
Two-way ANOVA				
Source of Variation	% of total variation	P value		
Interaction	6.42	< 0.0001		
treatment	2.74	< 0.0001		
region	75.47	< 0.0001		
Source of Variation	P value summary	Significant?		
Interaction	****	Yes		
treatment	****	Yes		
region	****	Yes		
Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	8	0.03396	0.004246	6.071
treatment	1	0.01450	0.01450	20.74
region	8	0.3991	0.04989	71.35
Residual	114	0.07972	0.0006993	

Parameter	
Table Analyzed	MS
Column A	veh
vs	vs
Column B	cort
Unpaired t test	
P value	0.4906
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=0.7079 df=14
How big is the difference?	
Mean ± SEM of column A	0.04063 ± 0.004595 N=8
Mean ± SEM of column B	0.04544 ± 0.005010 N=8
Difference between means	-0.004813 ± 0.006798
95% confidence interval	-0.01939 to 0.009769
R square	0.03456

Parameter	
Table Analyzed	Raphe
Column A	veh
vs	vs
Column B	cort
Unpaired t test	
P value	< 0.0001
P value summary	****
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=5.582 df=14
How big is the difference?	
Mean ± SEM of column A	0.03926 ± 0.005107 N=8
Mean ± SEM of column B	0.08344 ± 0.006045 N=8
Difference between means	-0.04418 ± 0.007914
95% confidence interval	-0.06115 to -0.02720
R square	0.6900

Parameter	
Table Analyzed	Dhipp
Column A	veh
vs	vs
Column B	cort
Unpaired t test	
P value	0.6119
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=0.5189 df=14
How big is the difference?	
Mean ± SEM of column A	0.02918 ± 0.003313 N=8
Mean ± SEM of column B	0.02678 ± 0.003228 N=8
Difference between means	0.0024 ± 0.004625
95% confidence interval	-0.007521 to 0.01232
R square	0.01887

Parameter	
Table Analyzed	LSD
Column A	veh
vs	vs
Column B	cort
Unpaired t test	
P value	0.0008
P value summary	***
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=4.273 df=14
How big is the difference?	
Mean ± SEM of column A	0.07584 ± 0.01298 N=8
Mean ± SEM of column B	0.1532 ± 0.01263 N=8
Difference between means	-0.07736 ± 0.01811
95% confidence interval	-0.1162 to -0.03852
R square	0.5660

Parameter	
Table Analyzed	insular cortex
Column A	veh
vs	vs
Column B	cort
Unpaired t test	
P value	0.9729
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=0.03455 df=14
How big is the difference?	
Mean ± SEM of column A	0.02551 ± 0.002094 N=8
Mean ± SEM of column B	0.02539 ± 0.002950 N=8
Difference between means	0.0001250 ± 0.003617
95% confidence interval	-0.007634 to 0.007884
R square	8.528e-005

Parameter	
Table Analyzed	amygdala
Column A	veh
vs	vs
Column B	cort
Unpaired t test	
P value	0.0962
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=1.783 df=14
How big is the difference?	
Mean ± SEM of column A	0.1719 ± 0.02137 N=8
Mean ± SEM of column B	0.2233 ± 0.01927 N=8
Difference between means	-0.05131 ± 0.02877
95% confidence interval	-0.1130 to 0.01041
R square	0.1851

Parameter	
Table Analyzed	vhipp
Column A	veh
vs	vs
Column B	cort
Unpaired t test	
P value	0.0476
P value summary	*
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=2.292 df=9
How big is the difference?	
Mean ± SEM of column A	0.03383 ± 0.007683 N=6
Mean ± SEM of column B	0.0574 ± 0.006416 N=5
Difference between means	-0.02357 ± 0.01028
95% confidence interval	-0.04682 to -0.0003128
R square	0.3687

Table Analyzed	subiculum
Column A	veh
vs	vs
Column B	cort
Unpaired t test	
P value	0.0924
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=1.948 df=7
How big is the difference?	
Mean ± SEM of column A	0.0316 ± 0.009003 N=5
Mean ± SEM of column B	0.05575 ± 0.008004 N=4
Difference between means	-0.02415 ± 0.01240
95% confidence interval	-0.05346 to 0.005165
R square	0.3516

Vasopressin 1a receptor autoradiography

Table Analyzed	veh vs cort layout-use this one			
Two-way ANOVA				
Source of Variation	% of total variation	P value		
Interaction	9.33	0.0006		
treatment	3.73	0.0002		
regions	42.27	< 0.0001		
Source of Variation	P value summary	Significant?		
Interaction	***	Yes		
treatment	***	Yes		
regions	****	Yes		
Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	11	0.2140	0.01945	3.192
treatment	1	0.08561	0.08561	14.05
regions	11	0.9690	0.08809	14.46
Residual	168	1.024	0.006094	

Parameter	
Table Analyzed	LDDM
Column A	veh
vs	vs
Column B	cort
Unpaired t test	
P value	0.6717
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=0.4328 df=14
How big is the difference?	
Mean ± SEM of column A	0.2850 ± 0.03482 N=8
Mean ± SEM of column B	0.3078 ± 0.03949 N=8
Difference between means	-0.02279 ± 0.05265
95% confidence interval	-0.1357 to 0.09014
R square	0.01321

Parameter	
Table Analyzed	MO-DG
Column A	veh
vs	vs
Column B	cort
Unpaired t test	
P value	0.1738
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=1.433 df=14
How big is the difference?	
Mean ± SEM of column A	0.1562 ± 0.02165 N=8
Mean ± SEM of column B	0.1934 ± 0.01426 N=8
Difference between means	-0.03715 ± 0.02593
95% confidence interval	-0.09276 to 0.01846
R square	0.1279

Parameter	
Table Analyzed	PO-DG
Column A	veh
vs	vs
Column B	cort
Unpaired t test	
P value	0.0036
P value summary	**
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=3.486 df=14
How big is the difference?	
Mean ± SEM of column A	0.1430 ± 0.01933 N=8
Mean ± SEM of column B	0.2469 ± 0.02267 N=8
Difference between means	-0.1039 ± 0.02979
95% confidence interval	-0.1678 to -0.03996
R square	0.4647

Parameter	
Table Analyzed	PO
Column A	veh
vs	vs
Column B	cort
Unpaired t test	
P value	0.1603
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=1.483 df=14
How big is the difference?	
Mean ± SEM of column A	0.1933 ± 0.02440 N=8
Mean ± SEM of column B	0.2540 ± 0.03288 N=8
Difference between means	-0.06071 ± 0.04094
95% confidence interval	-0.1485 to 0.02711
R square	0.1357

Parameter	
Table Analyzed	VM
Column A	veh
vs	vs
Column B	cort
Unpaired t test	
P value	0.5727
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=0.5776 df=14
How big is the difference?	
Mean ± SEM of column A	0.2076 ± 0.02382 N=8
Mean ± SEM of column B	0.2235 ± 0.01397 N=8
Difference between means	-0.01595 ± 0.02762
95% confidence interval	-0.07519 to 0.04329
R square	0.02327

Parameter	
Table Analyzed	PEFLH
Column A	veh
vs	vs
Column B	cort
Unpaired t test	
P value	0.6572
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=0.4534 df=14
How big is the difference?	
Mean ± SEM of column A	0.2077 ± 0.03069 N=8
Mean ± SEM of column B	0.2254 ± 0.02386 N=8
Difference between means	-0.01763 ± 0.03887
95% confidence interval	-0.1010 to 0.06576
R square	0.01447

Parameter	
Table Analyzed	VMDH
Column A	veh
vs	vs
Column B	cort
Unpaired t test	
P value	0.0034
P value summary	**
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=3.524 df=14
How big is the difference?	
Mean ± SEM of column A	0.3771 ± 0.03655 N=8
Mean ± SEM of column B	0.2223 ± 0.02436 N=8
Difference between means	0.1548 ± 0.04392
95% confidence interval	0.06059 to 0.2490
R square	0.4701

Parameter	
Table Analyzed	GRDG
Column A	veh
vs	vs
Column B	cort
Unpaired t test	
P value	0.0964
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=1.782 df=14
How big is the difference?	
Mean ± SEM of column A	0.2470 ± 0.03423 N=8
Mean ± SEM of column B	0.3439 ± 0.04224 N=8
Difference between means	-0.0969 ± 0.05437
95% confidence interval	-0.2135 to 0.01973
R square	0.1849

Parameter	
Table Analyzed	Septal nuclei
Column A	veh
vs	vs
Column B	cort
Unpaired t test	
P value	0.0026
P value summary	**
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=3.650 df=14
How big is the difference?	
Mean ± SEM of column A	0.3294 ± 0.02082 N=8
Mean ± SEM of column B	0.4290 ± 0.01762 N=8
Difference between means	-0.09957 ± 0.02728
95% confidence interval	-0.1581 to -0.04106
R square	0.4877

Parameter	
Table Analyzed	nucleus accumbens
Column A	veh
vs	vs
Column B	cort
Unpaired t test	
P value	0.1560
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=1.499 df=14
How big is the difference?	
Mean ± SEM of column A	0.3879 ± 0.03036 N=8
Mean ± SEM of column B	0.4496 ± 0.02782 N=8
Difference between means	-0.06175 ± 0.04118
95% confidence interval	-0.1501 to 0.02658
R square	0.1384

Parameter	
Table Analyzed	raphe
Column A	veh
vs	vs
Column B	cort
Unpaired t test	
P value	0.0140
P value summary	*
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=2.808 df=14
How big is the difference?	
Mean ± SEM of column A	0.2524 ± 0.02158 N=8
Mean ± SEM of column B	0.3378 ± 0.02142 N=8
Difference between means	-0.08538 ± 0.03041
95% confidence interval	-0.1506 to -0.02015
R square	0.3602

Parameter	
Table Analyzed	CeA
Column A	veh
vs	vs
Column B	cort
Unpaired t test	
P value	0.1742
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=1.431 df=14
How big is the difference?	
Mean ± SEM of column A	0.2084 ± 0.03316 N=8
Mean ± SEM of column B	0.2683 ± 0.02552 N=8
Difference between means	-0.05989 ± 0.04184
95% confidence interval	-0.1496 to 0.02986
R square	0.1277

Oxytocin tissue RIA

Parameter				
Table Analyzed	Stats relayout			
Two-way ANOVA				
Source of Variation	% of total variation	P value		
Interaction	18.23	< 0.0001		
treatment	2.69	< 0.0001		
region	58.73	< 0.0001		
Source of Variation	P value summary	Significant?		
Interaction	****	Yes		
treatment	****	Yes		
region	****	Yes		
Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	7	50880	7269	16.43
treatment	1	7517	7517	16.99
region	7	163938	23420	52.94
Residual	94	41587	442.4	

Parameter	
Table Analyzed	Hypothalamus
Column A	Vehicle
vs	vs
Column B	Corticosterone
Unpaired t test	
P value	0.0019
P value summary	**
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=4.047 df=11
How big is the difference?	
Mean ± SEM of column A	57.83 ± 17.30 N=6
Mean ± SEM of column B	191.3 ± 26.58 N=7
Difference between means	-133.5 ± 32.98
95% confidence interval	-206.1 to -60.89
R square	0.5982

Parameter	
Table Analyzed	Prefrontal cortex
Column A	Vehicle
vs	vs
Column B	Corticosterone
Unpaired t test	
P value	0.7603
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=0.3121 df=12
How big is the difference?	
Mean ± SEM of column A	0.6326 ± 0.1513 N=7
Mean ± SEM of column B	0.7277 ± 0.2643 N=7
Difference between means	-0.09506 ± 0.3045
95% confidence interval	-0.7586 to 0.5685
R square	0.008054

Parameter	
Table Analyzed	Septal nuclei
Column A	Vehicle
vs	vs
Column B	Corticosterone
Unpaired t test	
P value	0.3354
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=1.000 df=13
How big is the difference?	
Mean ± SEM of column A	8.684 ± 1.728 N=7
Mean ± SEM of column B	11.55 ± 2.204 N=8
Difference between means	-2.863 ± 2.862
95% confidence interval	-9.044 to 3.319
R square	0.07146

Parameter	
Table Analyzed	Amygdala
Column A	Vehicle
vs	vs
Column B	Corticosterone
Unpaired t test	
P value	0.3506
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=0.9748 df=11
How big is the difference?	
Mean ± SEM of column A	3.435 ± 1.345 N=6
Mean ± SEM of column B	2.121 ± 0.4962 N=7
Difference between means	1.314 ± 1.348
95% confidence interval	-1.653 to 4.281
R square	0.07951

Parameter	
Table Analyzed	Dorsal hippocampus
Column A	Vehicle
vs	vs
Column B	Corticosterone
Unpaired t test	
P value	0.3491
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=0.9780 df=11
How big is the difference?	
Mean ± SEM of column A	0.2225 ± 0.07956 N=5
Mean ± SEM of column B	0.3642 ± 0.1021 N=8
Difference between means	-0.1417 ± 0.1449
95% confidence interval	-0.4606 to 0.1772
R square	0.08000

Parameter	
Table Analyzed	Ventral hippocampus
Column A	Vehicle
vs	vs
Column B	Corticosterone
Unpaired t test	
P value	0.1257
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=1.636 df=13
How big is the difference?	
Mean ± SEM of column A	0.5763 ± 0.1209 N=6
Mean ± SEM of column B	0.3446 ± 0.08339 N=9
Difference between means	0.2318 ± 0.1416
95% confidence interval	-0.07418 to 0.5377
R square	0.1708

Parameter	
Table Analyzed	Raphe
Column A	Vehicle
vs	vs
Column B	Corticosterone
Unpaired t test	
P value	0.3797
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=0.9152 df=11
How big is the difference?	
Mean ± SEM of column A	8.176 ± 2.128 N=6
Mean ± SEM of column B	13.76 ± 5.312 N=7
Difference between means	-5.580 ± 6.097
95% confidence interval	-19.00 to 7.839
R square	0.07076

Parameter	
Table Analyzed	Plasma
Column A	Vehicle
vs	vs
Column B	Corticosterone
Unpaired t test	
P value	0.2538
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=1.204 df=11
How big is the difference?	
Mean ± SEM of column A	16.83 ± 4.695 N=7
Mean ± SEM of column B	10.01 ± 2.656 N=6
Difference between means	6.813 ± 5.658
95% confidence interval	-5.640 to 19.26
R square	0.1165

Vasopressin tissue content (RIA)

Parameter				
Table Analyzed	stats relayout			
Two-way ANOVA				
Source of Variation	% of total variation	P value		
Interaction	0.77	0.2175		
treatment	0.01	0.7391		
region	86.37	< 0.0001		
Source of Variation	P value summary	Significant?		
Interaction	ns	No		
treatment	ns	No		
region	****	Yes		
Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	7	3834	547.8	1.388
treatment	1	43.99	43.99	0.1115
region	7	428805	61258	155.2
Residual	110	43409	394.6	

Parameter	
Table Analyzed	Pituitary gland
Column A	Vehicle
vs	vs
Column B	Corticosterone
Unpaired t test	
P value	0.7713
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=0.2964 df=14
How big is the difference?	
Mean ± SEM of column A	53.35 ± 8.334 N=8
Mean ± SEM of column B	50.08 ± 7.250 N=8
Difference between means	3.274 ± 11.05
95% confidence interval	-20.42 to 26.97
R square	0.006237

Parameter	
Table Analyzed	Prefrontal cortex
Column A	Vehicle
vs	vs
Column B	Corticosterone
Unpaired t test	
P value	0.7557
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=0.3169 df=15
How big is the difference?	
Mean ± SEM of column A	1.341 ± 0.2826 N=7
Mean ± SEM of column B	1.236 ± 0.1936 N=10
Difference between means	0.1046 ± 0.3300
95% confidence interval	-0.5987 to 0.8079
R square	0.006650

Parameter	
Table Analyzed	Septal nuclei
Column A	Vehicle
vs	vs
Column B	Corticosterone
Unpaired t test	
P value	0.3891
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=0.8967 df=11
How big is the difference?	
Mean ± SEM of column A	9.428 ± 2.237 N=6
Mean ± SEM of column B	11.95 ± 1.760 N=7
Difference between means	-2.518 ± 2.808
95% confidence interval	-8.699 to 3.663
R square	0.06812

Parameter	
Table Analyzed	Amygdala
Column A	Vehicle
vs	vs
Column B	Corticosterone
Unpaired t test	
P value	0.0537
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=2.121 df=13
How big is the difference?	
Mean ± SEM of column A	5.085 ± 1.103 N=6
Mean ± SEM of column B	9.300 ± 1.435 N=9
Difference between means	-4.215 ± 1.987
95% confidence interval	-8.507 to 0.07694
R square	0.2571

Parameter	
Table Analyzed	Dorsal hippocampus
Column A	Vehicle
vs	vs
Column B	Corticosterone
Unpaired t test	
P value	0.5052
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=0.6839 df=14
How big is the difference?	
Mean ± SEM of column A	2.062 ± 0.7749 N=6
Mean ± SEM of column B	1.581 ± 0.2958 N=10
Difference between means	0.4803 ± 0.7022
95% confidence interval	-1.026 to 1.986
R square	0.03233

Parameter	
Table Analyzed	Ventral hippocampus
Column A	Vehicle
vs	vs
Column B	Corticosterone
Unpaired t test	
P value	0.1753
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=1.418 df=16
How big is the difference?	
Mean ± SEM of column A	3.674 ± 1.125 N=8
Mean ± SEM of column B	2.165 ± 0.3253 N=10
Difference between means	1.509 ± 1.064
95% confidence interval	-0.7462 to 3.764
R square	0.1117

Parameter	
Table Analyzed	Raphe
Column A	Vehicle
vs	vs
Column B	Corticosterone
Unpaired t test	
P value	0.2623
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=1.176 df=12
How big is the difference?	
Mean ± SEM of column A	68.27 ± 20.27 N=7
Mean ± SEM of column B	40.92 ± 11.37 N=7
Difference between means	27.34 ± 23.25
95% confidence interval	-23.31 to 77.99
R square	0.1034

Parameter	
Table Analyzed	Plasma
Column A	Vehicle
vs	vs
Column B	Corticosterone
Unpaired t test	
P value	0.2823
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=1.115 df=15
How big is the difference?	
Mean ± SEM of column A	172.5 ± 12.69 N=7
Mean ± SEM of column B	188.9 ± 8.528 N=10
Difference between means	-16.37 ± 14.68
95% confidence interval	-47.64 to 14.91
R square	0.07655

Plasma CORT and tissue oxytocin correlation – Vehicle treated group

	hypothalamus	prefrontal cortex	septal nuclei	amygdala	dorsal hippocampus	ventral hippocampus	raphe
Number of XY Pairs	5	7	7	6	5	5	6
Pearson r	0.08548	0.3317	0.2519	0.2107	0.1371	0.08744	0.1716
95% confidence interval	-0.8619 to 0.8999	-0.5618 to 0.8680	-0.6187 to 0.8448	-0.7249 to 0.8731	-0.8478 to 0.9094	-0.8614 to 0.9003	-0.7437 to 0.8630
P value (two-tailed)	0.8913	0.4674	0.5859	0.6886	0.8260	0.8888	0.7452
P value summary	ns	ns	ns	ns	ns	ns	ns
Is the correlation significant? (alpha=0.05)	No	No	No	No	No	No	No
R square	0.007307	0.1100	0.06343	0.04439	0.01879	0.007646	0.02943

Plasma CORT and tissue oxytocin correlation – CORT treated group

	hypothalamus	prefrontal cortex	septal nuclei	amygdala	dorsal hippocampus	ventral hippocampus	raphe
Number of XY Pairs	6	7	7	8	8	8	8
Pearson r	-0.01458	-0.6178	-0.1508	-0.3480	-0.5356	-0.3153	-0.03550
95% confidence interval	-0.8166 to 0.8066	-0.9356 to 0.2532	-0.8118 to 0.6795	-0.8454 to 0.4727	-0.9005 to 0.2717	-0.8346 to 0.5008	-0.7222 to 0.6865
P value (two-tailed)	0.9781	0.1393	0.7469	0.3982	0.1712	0.4469	0.9335
P value summary	ns	ns	ns	ns	ns	ns	ns
Is the correlation significant? (alpha=0.05)	No	No	No	No	No	No	No
R square	0.0002126	0.3816	0.02275	0.1211	0.2869	0.09939	0.001260

Plasma CORT and tissue vasopressin correlation – Vehicle treated group

Parameter	Pituitary gland	prefrontal cortex	septal nuclei	amygdala	dorsal hippocampus	ventral hippocampus	raphe
Number of XY Pairs	7	7	5	6	5	7	7
Pearson r	0.8099	0.2979	0.07844	0.6514	-0.2900	0.1268	-0.1890
95% confidence interval	0.1454 to 0.9709	-0.5870 to 0.8585	-0.8637 to 0.8986	-0.3400 to 0.9571	-0.9335 to 0.7960	-0.6925 to 0.8033	-0.8248 to 0.6578
P value (two-tailed)	0.0272	0.5164	0.9002	0.1611	0.6360	0.7864	0.6848
P value summary	*	ns	ns	ns	ns	ns	ns
Is the correlation significant? (alpha=0.05)	Yes	No	No	No	No	No	No
R square	0.6559	0.08874	0.006153	0.4243	0.08412	0.01608	0.03572

Plasma CORT and tissue vasopressin correlation – CORT treated group

Parameter	Pituitary gland	prefrontal cortex	septal nuclei	amygdala	dorsal hippocampus	ventral hippocampus	raphe
Number of XY Pairs	7	9	7	9	9	9	6
Pearson r	0.6058	-0.3480	0.2985	0.07275	-0.1153	-0.1967	0.02719
95% confidence interval	-0.2710 to 0.9332	-0.8222 to 0.4113	-0.5865 to 0.8586	-0.6215 to 0.7030	-0.7241 to 0.5945	-0.7614 to 0.5378	-0.8022 to 0.8207
P value (two-tailed)	0.1494	0.3588	0.5155	0.8525	0.7678	0.6120	0.9592
P value summary	ns	ns	ns	ns	ns	ns	ns
Is the correlation significant? (alpha=0.05)	No	No	No	No	No	No	No
R square	0.3670	0.1211	0.08911	0.005292	0.01329	0.03868	0.0007391

Plasma oxytocin and tissue oxytocin – Vehicle treated group

Parameter	hypothalamus	prefrontal cortex	septal nuclei	amygdala	dorsal hippocampus	ventral hippocampus	raphe
Number of XY Pairs	5	6	6	5	5	5	5
Pearson r	-0.8715	-0.3386	-0.2208	-0.3645	-0.2798	-0.05288	-0.5926
95% confidence interval	-0.9915 to 0.04688	-0.9023 to 0.6524	-0.8756 to 0.7199	-0.9434 to 0.7633	-0.9320 to 0.8001	-0.8935 to 0.8701	-0.9685 to 0.6073
P value (two-tailed)	0.0542	0.5116	0.6741	0.5464	0.6484	0.9327	0.2923
P value summary	ns	ns	ns	ns	ns	ns	ns
Is the correlation significant? (alpha=0.05)	No	No	No	No	No	No	No
R square	0.7595	0.1146	0.04876	0.1329	0.07830	0.002796	0.3512

Plasma oxytocin and tissue oxytocin – CORT treated group

Parameter	hypothalamus	prefrontal cortex	septal nuclei	amygdala	dorsal hippocampus	ventral hippocampus	raphe
Number of XY Pairs	3	4	5	6	5	6	5
Pearson r	-0.9569	-0.5573	0.08840	0.3021	0.3084	-0.004846	-0.1889
95% confidence interval	0.1875	-0.9888 to 0.8696	-0.8611 to 0.9005	-0.6751 to 0.8944	-0.7885 to 0.9360	-0.8133 to 0.8100	-0.9182 to 0.8321
P value (two-tailed)	ns	0.4427	0.8876	0.5607	0.6136	0.9927	0.7609
P value summary	No	ns	ns	ns	ns	ns	ns
Is the correlation significant? (alpha=0.05)	0.9157	No	No	No	No	No	No
R square		0.3106	0.007814	0.09125	0.09511	2.348e-005	0.03569

Plasma vasopressin and tissue vasopressin – Vehicle treated group

Parameter	Pituitary gland	prefrontal cortex	septal nuclei	amygdala	dorsal hippocampus	ventral hippocampus	raphe
Number of XY Pairs	7	6	5	5	5	7	6
Pearson r	-0.3861	0.2248	0.5953	0.4189	-0.4880	-0.2183	0.8532
95% confidence interval	-0.8826 to 0.5176	-0.7179 to 0.8765	-0.6046 to 0.9688	-0.7352 to 0.9501	-0.9579 to 0.6926	-0.8343 to 0.6401	0.1352 to 0.9837
P value (two-tailed)	0.3923	0.6685	0.2895	0.4827	0.4043	0.6381	0.0307
P value summary	ns	ns	ns	ns	ns	ns	*
Is the correlation significant? (alpha=0.05)	No	No	No	No	No	No	Yes
R square	0.1491	0.05052	0.3544	0.1755	0.2381	0.04768	0.7280

Plasma vasopressin and tissue vasopressin – CORT treated group

Parameter	Pituitary gland	prefrontal cortex	septal nuclei	amygdala	dorsal hippocampus	ventral hippocampus	raphe
Number of XY Pairs	8	10	7	9	10	10	7
Pearson r	0.3861	0.2748	0.2291	-0.2152	-0.4061	-0.3406	-0.7133
95% confidence interval	-0.4378 to 0.8575	-0.4292 to 0.7711	-0.6333 to 0.8377	-0.7694 to 0.5239	-0.8249 to 0.3005	-0.7990 to 0.3681	-0.9540 to 0.08621
P value (two-tailed)	0.3448	0.4422	0.6212	0.5782	0.2442	0.3355	0.0719
P value summary	ns	ns	ns	ns	ns	ns	ns
Is the correlation significant? (alpha=0.05)	No	No	No	No	No	No	No
R square	0.1491	0.07552	0.05249	0.04630	0.1649	0.1160	0.5087

t=0hrs vs t=24hrs

Parameter				
Table Analyzed	VEHwater consumed (ml/rat)			
Two-way ANOVA				
Source of Variation	% of total variation	P value		
Interaction	8.43	0.5615		
treatment	3.34	0.0844		
Duration	23.22	0.0224		
Source of Variation	P value summary	Significant?		
Interaction	ns	No		
treatment	ns	No		
Duration	*	Yes		
Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	9	274.6	30.51	0.8643
treatment	1	108.7	108.7	3.079
Duration	9	756.6	84.07	2.381
Residual	60	2118	35.30	

Parameter				
Table Analyzed	CORTwater consumed (ml/rat)			
Two-way ANOVA				
Source of Variation	% of total variation	P value		
Interaction	8.39	0.3070		
treatment	0.52	0.4141		
Duration	44.82	< 0.0001		
Source of Variation	P value summary	Significant?		
Interaction	ns	No		
treatment	ns	No		
Duration	****	Yes		
Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	9	305.7	33.97	1.208
treatment	1	19.01	19.01	0.6763
Duration	9	1634	181.5	6.458
Residual	60	1687	28.11	

Parameter				
Table Analyzed	VEHbody weight (g)			
Two-way ANOVA				
Source of Variation	% of total variation	P value		
Interaction	0.55	0.8666		
treatment	0.85	0.0044		
Day	62.04	< 0.0001		
Source of Variation	P value summary	Significant?		
Interaction	ns	No		
treatment	**	Yes		
Day	****	Yes		
Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	10	1353	135.3	0.5321
treatment	1	2104	2104	8.273
Day	10	152859	15286	60.11
Residual	242	61542	254.3	

Parameter				
Table Analyzed	CORTbody weight (g)			
Two-way ANOVA				
Source of Variation	% of total variation	P value		
Interaction	1.10	0.9674		
treatment	9.67	< 0.0001		
duration	5.22	0.0943		
Source of Variation	P value summary	Significant?		
Interaction	ns	No		
treatment	****	Yes		
duration	ns	No		
Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	10	847.9	84.79	0.3461
treatment	1	7465	7465	30.47
duration	10	4029	402.9	1.644
Residual	264	64682	245.0	

Parameter	
Table Analyzed	adrenal (mg)/100 g body weight (g)
Column A	VEHICLE
vs	vs
Column B	CORT
Unpaired t test	
P value	< 0.0001
P value summary	****
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=7.078 df=15
How big is the difference?	
Mean ± SEM of column A	12.88 ± 1.050 N=8
Mean ± SEM of column B	4.127 ± 0.7018 N=9
Difference between means	8.755 ± 1.237
95% confidence interval	6.119 to 11.39
R square	0.7696

Parameter	
Table Analyzed	thymus (mg)/100 g body weight (g)
Column A	VEHICLE
vs	vs
Column B	CORT
Unpaired t test	
P value	< 0.0001
P value summary	****
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=9.009 df=16
How big is the difference?	
Mean ± SEM of column A	170.9 ± 7.931 N=8
Mean ± SEM of column B	87.65 ± 5.329 N=10
Difference between means	83.29 ± 9.245
95% confidence interval	63.69 to 102.9
R square	0.8353

CB1 receptor autoradiography

Parameter				
Table Analyzed	fmol/mg tiss all			
Two-way ANOVA				
Source of Variation	% of total variation	P value		
Interaction	0.86	0.6498		
treatment	0.22	0.0499		
region	84.80	< 0.0001		
Source of Variation	P value summary	Significant?		
Interaction	ns	No		
treatment	*	Yes		
region	****	Yes		
Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	18	2222	123.4	0.8418
treatment	1	569.6	569.6	3.884
region	18	218766	12154	82.88
Residual	248	36367	146.6	

Parameter	
Table Analyzed	ttest striata
Column A	vehicle
vs	vs
Column B	cort
Unpaired t test	
P value	0.0002
P value summary	***
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=5.040 df=14
How big is the difference?	
Mean ± SEM of column A	36.53 ± 2.190 N=8
Mean ± SEM of column B	23.85 ± 1.238 N=8
Difference between means	12.68 ± 2.515
95% confidence interval	7.281 to 18.07
R square	0.6447

Parameter	
Table Analyzed	ttest vhip
Column A	vehicle
vs	vs
Column B	cort
Unpaired t test	
P value	0.6053
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=0.5287 df=14
How big is the difference?	
Mean ± SEM of column A	30.74 ± 3.239 N=8
Mean ± SEM of column B	32.80 ± 2.164 N=8
Difference between means	-2.059 ± 3.895
95% confidence interval	-10.41 to 6.296
R square	0.01958

Parameter	
Table Analyzed	ttest SNR
Column A	vehicle
vs	vs
Column B	cort
Unpaired t test	
P value	0.4580
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=0.7632 df=14
How big is the difference?	
Mean ± SEM of column A	147.5 ± 19.72 N=8
Mean ± SEM of column B	130.0 ± 11.86 N=8
Difference between means	17.56 ± 23.01
95% confidence interval	-31.79 to 66.91
R square	0.03994

Parameter	
Table Analyzed	ttest Dhipp CA1
Column A	vehicle
vs	vs
Column B	cort
Unpaired t test	
P value	0.7596
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=0.3120 df=14
How big is the difference?	
Mean ± SEM of column A	24.16 ± 1.605 N=8
Mean ± SEM of column B	25.01 ± 2.215 N=8
Difference between means	-0.8533 ± 2.735
95% confidence interval	-6.720 to 5.013
R square	0.006906

Parameter	
Table Analyzed	ttest Dhipp CA2
Column A	vehicle
vs	vs
Column B	cort
Unpaired t test	
P value	0.7438
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=0.3333 df=14
How big is the difference?	
Mean ± SEM of column A	26.10 ± 1.393 N=8
Mean ± SEM of column B	27.14 ± 2.778 N=8
Difference between means	-1.036 ± 3.108
95% confidence interval	-7.702 to 5.630
R square	0.007873

Parameter	
Table Analyzed	ttest Dhipp CA3
Column A	vehicle
vs	vs
Column B	cort
Unpaired t test	
P value	0.5152
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=0.6677 df=14
How big is the difference?	
Mean ± SEM of column A	27.43 ± 2.114 N=8
Mean ± SEM of column B	25.13 ± 2.719 N=8
Difference between means	2.299 ± 3.444
95% confidence interval	-5.088 to 9.687
R square	0.03086

Parameter	
Table Analyzed	ttest PODG
Column A	vehicle
vs	vs
Column B	cort
Unpaired t test	
P value	0.8281
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=0.2213 df=14
How big is the difference?	
Mean ± SEM of column A	21.32 ± 1.336 N=8
Mean ± SEM of column B	21.93 ± 2.404 N=8
Difference between means	-0.6086 ± 2.751
95% confidence interval	-6.508 to 5.291
R square	0.003484

Parameter	
Table Analyzed	ttest Raphe
Column A	vehicle
vs	vs
Column B	cort
Unpaired t test	
P value	0.0008
P value summary	***
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=4.227 df=14
How big is the difference?	
Mean ± SEM of column A	16.55 ± 1.796 N=8
Mean ± SEM of column B	8.862 ± 0.2834 N=8
Difference between means	7.684 ± 1.818
95% confidence interval	3.784 to 11.58
R square	0.5606

Parameter	
Table Analyzed	ttest Entorhinal cortex
Column A	vehicle
vs	vs
Column B	cort
Unpaired t test	
P value	0.2338
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=1.244 df=14
How big is the difference?	
Mean ± SEM of column A	18.44 ± 2.004 N=8
Mean ± SEM of column B	15.43 ± 1.358 N=8
Difference between means	3.012 ± 2.421
95% confidence interval	-2.180 to 8.205
R square	0.09959

Parameter	
Table Analyzed	ttest PrL
Column A	vehicle
vs	vs
Column B	cort
Unpaired t test	
P value	0.7824
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=0.2816 df=14
How big is the difference?	
Mean ± SEM of column A	23.70 ± 2.064 N=8
Mean ± SEM of column B	22.85 ± 2.184 N=8
Difference between means	0.8461 ± 3.005
95% confidence interval	-5.599 to 7.291
R square	0.005633

Parameter	
Table Analyzed	ttest CgL
Column A	vehicle
vs	vs
Column B	cort
Unpaired t test	
P value	0.8798
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=0.1540 df=14
How big is the difference?	
Mean ± SEM of column A	26.64 ± 1.250 N=8
Mean ± SEM of column B	26.98 ± 1.766 N=8
Difference between means	-0.3333 ± 2.164
95% confidence interval	-4.974 to 4.308
R square	0.001691

Parameter	
Table Analyzed	ttest M1
Column A	vehicle
vs	vs
Column B	cort
Unpaired t test	
P value	0.0112
P value summary	*
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=2.920 df=14
How big is the difference?	
Mean ± SEM of column A	35.53 ± 3.440 N=8
Mean ± SEM of column B	24.02 ± 1.927 N=8
Difference between means	11.51 ± 3.943
95% confidence interval	3.055 to 19.97
R square	0.3785

Parameter	
Table Analyzed	ttest M2
Column A	vehicle
vs	vs
Column B	cort
Unpaired t test	
P value	0.1760
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=1.425 df=14
How big is the difference?	
Mean ± SEM of column A	30.83 ± 1.630 N=8
Mean ± SEM of column B	27.20 ± 1.960 N=8
Difference between means	3.632 ± 2.549
95% confidence interval	-1.835 to 9.099
R square	0.1267

Parameter	
Table Analyzed	ttest nucc shell
Column A	vehicle
vs	vs
Column B	cort
Unpaired t test	
P value	0.2291
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=1.258 df=14
How big is the difference?	
Mean ± SEM of column A	15.05 ± 1.947 N=8
Mean ± SEM of column B	18.09 ± 1.432 N=8
Difference between means	-3.039 ± 2.417
95% confidence interval	-8.223 to 2.145
R square	0.1015

Parameter	
Table Analyzed	ttest nucc core
Column A	vehicle
vs	vs
Column B	cort
Unpaired t test	
P value	0.8780
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=0.1564 df=14
How big is the difference?	
Mean ± SEM of column A	15.05 ± 1.947 N=8
Mean ± SEM of column B	15.42 ± 1.328 N=8
Difference between means	-0.3685 ± 2.356
95% confidence interval	-5.423 to 4.686
R square	0.001744

Parameter	
Table Analyzed	ttest amygdala
Column A	vehicle
vs	vs
Column B	cort
Unpaired t test	
P value	0.9644
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=0.04548 df=14
How big is the difference?	
Mean ± SEM of column A	14.18 ± 0.6574 N=8
Mean ± SEM of column B	14.24 ± 1.156 N=8
Difference between means	-0.06048 ± 1.330
95% confidence interval	-2.913 to 2.792
R square	0.0001477

Parameter	
Table Analyzed	ttest VMDH
Column A	vehicle
vs	vs
Column B	cort
Unpaired t test	
P value	0.2456
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=1.253 df=8
How big is the difference?	
Mean ± SEM of column A	10.69 ± 1.958 N=5
Mean ± SEM of column B	7.955 ± 0.9608 N=5
Difference between means	2.732 ± 2.181
95% confidence interval	-2.297 to 7.762
R square	0.1640

Parameter	
Table Analyzed	ttest PVN
Column A	vehicle
vs	vs
Column B	cort
Unpaired t test	
P value	0.0841
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=1.972 df=8
How big is the difference?	
Mean ± SEM of column A	9.101 ± 0.6733 N=5
Mean ± SEM of column B	7.013 ± 0.8176 N=5
Difference between means	2.088 ± 1.059
95% confidence interval	-0.3540 to 4.531
R square	0.3270

Parameter	
Table Analyzed	ttest subiculum
Column A	vehicle
vs	vs
Column B	cort
Unpaired t test	
P value	0.5896
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=0.5619 df=8
How big is the difference?	
Mean ± SEM of column A	29.52 ± 1.518 N=5
Mean ± SEM of column B	30.78 ± 1.656 N=5
Difference between means	-1.262 ± 2.246
95% confidence interval	-6.442 to 3.918
R square	0.03796

Appendix C

Brain/ Region	Width (mm)	Height (mm)
Prefrontal cortex		
PrL	0.25	1.00
CGL	0.25	1.00
M2	0.25	1.00
M1	0.25	1.00
Striata	1.00	1.00
Septal nuclei		
LSD	0.40	0.50
MSD	0.50	0.80
Nucleus Accumbens		
Shell	0.50	0.80
Core		
Insular cortex	0.30	1.00
Dorsal hippocampus		
CA1-rad	0.16	0.80
CA2-rad	0.15	0.60
CA2-pyr	0.15	0.60
CA3-rad	0.16	0.80
MODG	0.20	0.80
PODG	0.20	0.80
Thalamus		
LDDM	0.40	0.40
VM	1.00	1.00
PO	0.60	1.00
Hypothalamus		
VMDH	0.40	1.00
PEFLH	0.60	0.70
PVN	0.10	0.60
Amygdala		
CeA	0.60	0.60
Ventral hippocampus		
CA1 rad	0.35	2.00
Subiculum	0.30	0.60
GRDG	0.35	1.00
Piriform cortex	0.30	1.20
Entorhinal cortex	0.70	0.35
Raphé	0.50	0.25